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Microbial toxicity and bioreduction of antimony under anaerobic conditions

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Microbial toxicity and bioreduction of antimony under anaerobic conditions

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ABSTRACT

Antimony is a metalloid belonging to the group 15 in the periodic table. With the increasing use of antimony in recent years because of different industrial uses, the importance and the interest in this element is increasing, same as its release into the environment. Little is known about the environmental impacts of Sb and the potential involvement of microorganisms in its cycling.

The objectives of this study are three-fold: (i) to develope an analytical method to determine antimony speciation, (ii) to evaluate the microbial toxicity of Sb (V) and Sb (III), and (iii) to investigate the microbial reduction of Sb (V) by different inoculum sources under anaerobic conditions.

Batch experiments were performed to assess the inhibitory impact of the most common antimony species in the environment (antimonite (Sb (III)) and antimonate (Sb (V))) towards different microbial targets, and to investigate the ability of microorganisms in variuos inocula to reduce Sb (V) under anaerobic conditions and the kinetics of microbial Sb reduction.

A new method was developed to speciate antinomy that couples liquid chromatography to inductively coupled plasma – optical emission spectrometry (LC-ICP-OES). This new analytical methodology was shown to be a viable alternative to the more expensive/sophisticated method currently used for Sb speciation, i.e. LC-ICP-MS (liquid chromatography- inductively couple plasma – mass spectrometry). The LC-ICP-OES method developed can measure different metal Sb species in a single run. The analytical conditions, instrument setup and data processing are explained in detail in the Materials and Methods section and the Appendixes of this report. Possible chemical interferences during the analysis of Sb at the three recommended wavelengths are discussed in the Results chapter.

The results of toxicity tests showed that Sb (III) displays a higher inhibition to methanogenic microorganisms than Sb (V). The 50% inhibiting concentration (IC50) determined in methanogenic bioassays was 150 mg/L for Sb (V) and 10-12 mg/L for Sb (III). However, the inhibitory potential of Sb (V) was found to increase with increasing exposure time, likely due to the microbial conversion of Sb (V) to the more toxic Sb (III).

Five different inocula ("Mahou", "Eerbeek", "Rockteen", "INA", "Agua Caliente" and "Agua Nueva") were screened for their ability to reduce Sb (V) in anaerobic assays supplied with H_2 as the electron donor. Results showed that some anaerobic microorganisms are capable of reducing Sb (V) to Sb (III) and that, if there is enough sulfide in the solution, Sb (III) will precipitate as reddish particles.

These results demonstrate that microorganisms capable of reducing Sb (V) are widely distributed in the environment. Preliminary results indicate that microbial reduction of Sb in the presence of (biogenic) sulfide results in the formation of a highly insoluble compound (Sb_2S_3). This biomineralization reaction could provide the basis for developing a novel biotechnology process for the removal of Sb from contaminated water.

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1. INTRODUCTION

1.1. Antimony

1.1.1 Importance and applications

The importance of antimony as an industrial material is growing rapidly due to its different applications in new products. For instance, it is used in the semiconductor industry in the manufacture of infrared detectors and diodes (Filella M. et al. 2002a) . It also forms alloys used in the manufacture of automobile batteries, small arms bullets, cable sheathing, and solders. Antimony trioxide (Sb_2O_3) is the most important antimony compound for industries. It is used as a mordant, flame retardant, pigment, catalyst and PVC stabilizer. Some antimony compounds have been used to treat different digestive diseases (Abin and Hollibaugh 2014).

Nowadays the world production and reserves of antimony are estimated at about 160,000 and 1,800,000 tons, respectively. Most of it is extracted from China (about 80 % of the world extraction) (Li et al. 2016).

Table 1.1. Antimony production 2015

Country	Tonnes	% of total
China	115000	76.7
Russia	9000	6
Australia	5500	3.7
Bolivia	5000	3.3
Tajikistan	4700	3.1
Total world	150000	100

1.2. Chemistry

1.2.1 Oxidation states

Antimony (Sb) is a toxic element belonging to the group 15 of the periodic table and it is positioned right below arsenic (As). It exists in four oxidation starts (-III, 0, III and V) (Li et al. 2016). In the environment the most common solid form is stibnite (Sb_2S_3) (Nguyen and Lee 2014) and the most common forms in aqueous solutions are antimonate ($Sb(OH)^{6-}$, as Sb (V)) and antimonite ($Sb(OH)_3$, as Sb (III)). In oxygenated systems, antimonate is the predominant form, whereas in reducing environment the dominant form is antimonite (Li et al. 2016).

Inorganic antimonite methylation is important to the toxicity, bioaccumulation and mobility in the environment. There are several Sb organometallic compounds that can be formed due to microbial methylation of antimony; including monomethylstibine (MMS), dimethylstibine (DMS) and trimethylstibine (TMS) (Li et al. 2016).

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1.2.2 pKa, Solubility

Depending on the pH there are different predominant antimony forms. With very low pH the predominant form is SbO_2^+. When the pH raises to lightly acidic, neutral or basic the predominant species could be $[Sb(OH)_6]^-$ or SbO_3^-. Antimony pentoxide (Sb_2O_5) is hardly soluble in water. Also antimony trioxide (Sb_2O_3) has a relatively low solubility in water in the pH range 2-10 (Filella M. et al. 2002b).

1.2.3 Chemical oxidation and reduction of antimony

Abiotic dark oxidation of Sb (III) with O2 is extremely slow with a half-life of 170 years at pH 8.5. On the other hand, oxidation is much faster with H2O2 with a half-life of 118 days with 1 μM H2O2 at pH 8.0. Several other oxidants are capable to oxidize Sb (III) to Sb (V) such as Fe and Mn

oxyhydroxides, humic acids and iodate. Sb (III) can also be oxidized by photo-induced oxidation in natural surface waters. In some experiments done with seawater, various phytoplankton species increased the rate of this process. Light seem to improve the reduction process even with present microbes (Li et al. 2016).

Sb (V) can be chemically also be reduced to Sb (III) by sulfide and, if there is still enough sulfur, Sb (III) can form a highly insoluble compound stibnite (Sb2S3, solubility product= 1.6×10-93 (Polack et al. 2009)) and precipitate. This can only happen under anaerobic conditions because otherwise, under aerobic conditions, sulfide would precipitate and antimony would not precipitate. The Sb2S3 precipitate formed has a reddish color and it is formed in the range of pH from 5 to 8 (Zhang et al. 2016).

A report proved that Sb (V) from water coming from mine drainage at pH 5, 7 and 9 by precipitation with sulfide generated by sulfate reducing bacteria (SRB). The reduction mechanism involved (i) reduction of Sb (V) to Sb (III) by biogenic sulfide, (ii) precipitation of the reduced Sb (III) with sulfide as stibnite (Sb2S3). The maximum removal efficiency was reached at pH 7. After 7 days of treatment, the Sb (V) concentration was decreased from 20 mg/L to 0.16 mg/L at pH 7 and to 2.73 mg/L at pH 9. The Sb (III) concentration increased with time reaching maximum values of about 1 mg/L but remaining in the controls below 0.2 mg/L (Wang et al. 2013).

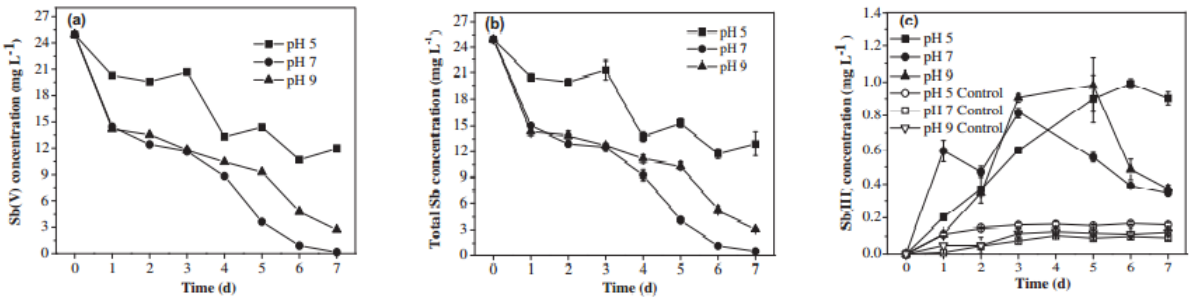

Figure 1.1. *Sb (V) reduction with SRB treatment (a), total antimony loss by SRB treatment (b) and solution pH with incubation time (c) (Wang et al. 2013).*

1.3. Antimony contamination

Antimony contamination can be caused by both natural (volcanism and the weathering of Sb-bearing rocks and minerals, for example) and anthropogenic activities (mining and smelting wastewater) (Li et al. 2016). To give a general idea of the problem caused by these antimony sources, in natural waters the typical concentration of antimony is less than 1 µg/L but in geothermal waters it can be more than 1 g/L. Antimony concentrations in water are also high in antimony-enriched mineralized areas (Filella M. et al. 2007). Acid mine drainage (AMD) generated in areas impacted by mining can also contain elevated concentrations of antimony. AMD is a water flux contaminated with high concentrations of sulfate and metals due to mining activities.

Volcanism has been reported to release about 5 tonnes of antimony per year to the atmosphere and this only represents about 3-5 % of antimony global emission (Smichowski 2008). Serious co-pollution of antimony and arsenic has been found in the soils, mine drainage, drinking water, crops and local biota around the largest antimony mine in the world (in China) reporting cases of acute arsine poisoning and that about 22.8% of the mine workers showed symptoms of antimony dermatitis (Wu et al. 2011).

1.4. Ecological effects

1.4.1. Legislation - Maximum contaminant level (MCL) in drinking water

Antimony shares some chemical and toxicological properties with arsenic. Antimony and its compounds are considered hazardous pollutants by both the U.S. Environmental Protection Agency (EPA) and the Council of European Communities (CEC). In fact, the EPA stipulated a lower maximum concentration permitted for antimony than for arsenic, reflecting its greater overall toxicity. The maximum contaminant level (MCL) is the legal threshold limit on the amount of a substance that is allowed in public water systems under the Safe Drinking Water Act. The MCL of Sb in drinking water is 6 µg/L according to the US-EPA (EPA. 1999) and the level established by the CEC is 5 µg/L (CEC. 1998).

1.4.2. Toxicity

Antimony is not an essential element in plants or animals. Antimony is known to be toxic to most living organisms at high concentrations (Nguyen and Lee 2014). Its bioavailability and toxicological effects depend on its oxidation state and chemical form (Terry et al. 2015). Similar to most trace metals, antimony toxicity strongly depends upon its chemical speciation. The general order of toxicity for antimony species is greater in Sb (III), followed by Sb (V) (Li et al. 2016). Sb (III)) is toxic to several aquatic organisms; for example using Sb (III) (antimony potassium tartrate), median 50% lethal concentrations (LC_{50}) of 4.9 mg/L and 261 mg/L have been reported for the planktonic crustacea, *Simocephalus mixtus* and larvae of *Ozyzias latipes*, respectively, after 24 h of exposure (Nam et al. 2009).

Little is known about microbial toxicity of antimony. It is suspected that it behaves similar than arsenic. One of the purposes of this research is to investigate the toxicity of antimony and how it varies depending on the Sb speciation.

1.4.3. Bioaccumulation / Uptake by plants

Antimony can be taken up by plants from the environment and due to the food chains this can ultimately become dangerous to human health. As was the case with arsenic, microorganisms appear to play an important role in the speciation and bioavailability of antimony (Li et al. 2016). Sb (III) has a higher tendency to bioaccumulate that Sb (V). For example, 20% of the total amount of Sb (III) bioaccumulated at initial low concentrations (about 5 mg/L) (Boriová et al. 2014).

Research with waterlogged *Lolium perenne* grass reported that Sb (III) was being absorbed by the grass (15% of the initial amount dosed). Comparison of the concentrations of Sb (III) by the roots and the concentration on the bottom of the liquid phase demonstrated higher accumulation in the roots zone (Wan et al. 2013).

1.5. Microbial transformation of Sb

There are different possibilities for microbial transformation of antimony. The key processes include biosorption, microbial reduction, microbial oxidation and methylation. These processes will be discussed briefly below.

1.5.1. Microbial recuperation/elimination of metals by biosorption

Microbes can adsorb different metals including Pt, Fe, Ni, Zn, etc. The metal binding capacities of microbes compare favorably with commercial ion exchangers. The interaction between metals and microbes can occur as absorption, ion exchange, complexation or precipitation. All this processes are affected by the chemical groups located on surfaces of microbial cells (Zhuang et al. 2015).

1.5.2. Microbial oxidation of antimony

Several studies have been performed about antimony oxidation due to microbes and there is an increasing interest on microbial interactions with antimony lately. For example, the microorganism *Stibiobacter senarmontii* was found to save energy produced by Sb (III) oxidation with oxygen to support chemoautotrophic growth. The strains used to oxidize Sb (III) usually grew heterotrophically (Li et al. 2016). In an article back in the 70s, *S. senarmontii* was reported to grow as autotroph using Sb (III) as electron donor under oxic conditions. The Sb (III) source was solid antimony trioxide (Sb_2S_3) and the oxidation products Sb (V) and Sb (III) oxides (Terry et al. 2015).

It has been reported that the wild type of *Agrobacterium tumefaciens* as well as two inner-membrane-bound oxidase mutant strains that could not oxidize As (III) were all capable of oxidizing Sb (III). These results suggest that Sb (III) oxidation and As (III) oxidation are catalyzed by different enzymatic pathways (Terry et al. 2015).

1.5.3. Microbial reduction of antimony

Very few studies have considered the ability of microorganisms to reduce antimony. A sporulating member of a deeply branching lineage within the order *Bacillales* (phylum *Firmicutes*) was capable of completely reducing completely reducing 243.5 mg/L of Sb (V) under aerobic conditions in about 80 h and at 30ºC using lactate as electron donor. This reaction was mediated by microbial activity because the authors did not observe any Sb (V) reduction in controls lacking lactate or microorganisms. At the same time that Sb (V) was reduced, it gradually appeared a white crystalline precipitate of Sb_2O_3 (Abin and Hollibaugh 2014).

In another experiment using an anoxic culture and hydrogen as electron donor, 80.36 mg/L of Sb (V) was reduced in 10 days under anaerobic conditions. The culture was not able to reduce the antimony in the absence of hydrogen, nor when it was autoclaved, demonstrating that the reaction was mediated by microorganism. When the concentration of Sb (V) present was reduced, the total soluble antimony concentration also decreased due to Sb (III) precipitation. The culture was also able to reduce Sb (V) using lactate as the electron donor albeit at a slower rate than when hydrogen was used as electron donor. The study demonstrated that the reduction of Sb was coupled to microbial growth, In this process the Sb (III) precipitation is supposed to be the limiting step for Sb removal (Lai et al. 2016). Moreover, a chemoautotrophic microorganism was shown to reduce Sb (V) with hydrogen as electron donor and form a precipitate of antimony trioxide (Sb_2S_3) (Li et al. 2016).

Microbial reduction of Sb (V) is a promising approach for the bioremediation of contaminated water due to the possibility of removing Sb (III) by precipitation with biogenic sulfide or by absorption to Fe phases in a reducing environment (Li et al. 2016).

1.5.4. Methylation of antimony

Some microorganisms can form methylantimony species. Sb methylation involves transformation of Sb (III) to MMS, which is further transformed to TMS. Methylation of antimony by some microorganisms (*Scopulariopsis brevicaulis*, *Cryptococcus humicolus*) is also enhanced by the presence of arsenic. On the other hand, arsenic methylation by both microorganisms was inhibited by the presence of antimony. The presence of arsenic did not only enhance antimony methylation

by *C. humicolus* but also influenced antimony speciation. The illustration below shows some possible microbial transformations of different Sb species (Li et al. 2016).

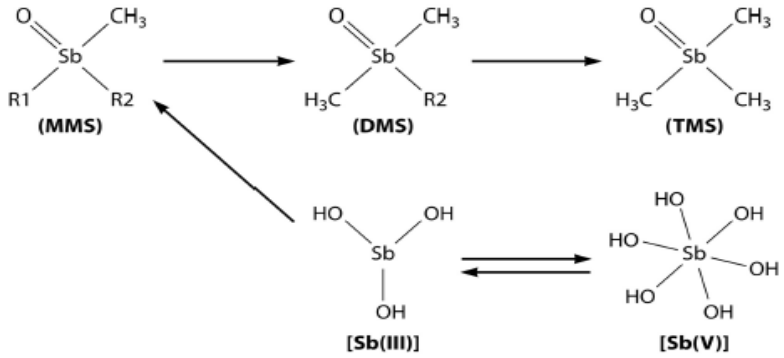

Figure 1.2. *Different possible antimony reaction pathways (Li et al. 2016).*

2. OBJECTIVES

The objectives of this research are threefold:

i) Develop an analytical method to determine antimony speciation.

ii) To evaluate the microbial toxicity of Sb (V) and Sb (III).

iii) To study the microbial reduction of Sb (V) by different inoculum sources under anaerobic conditions.

3. MATERIALS & METHODS

3.1. CHEMICALS

Sb (V) (potassium hexahydroxoantimonate ($KSb(OH)_6$, CAS # 12208-13-8, purity ≥ 99.0%) was obtained from Fluka Analytical and, Sb (III) (potassium antimony tartrate hydrate ($C_8H_4K_2O_{12}Sb_2 \cdot xH_2O$, CAS # 331753-56-1, purity ≥ 99.95%) or antimony (III) trichloride ($SbCl_3$, CAS # 10025-91-9, purity ≥ 99.95%)) were both obtained from Sigma-Aldrich Chemistry. Potassium antimony (III) tartrate was used in the toxicity studies.

3.2. BIOASSAYS

Different bioassays were carried out under different conditions depending on the objective aimed each time.

3.2.1. Inoculum

The sludge used in the toxicity studies was obtained from an anaerobic bioreactor treating brewery wastewater (M, Mahou, Guadalajara, Spain). Microbial reduction of Sb (V) was tested using six different inoculum sources, namely, Mahou (described above); anaerobic pond sediments (AC, Agua Caliente park, Tucson, AZ, USA); return activated sludge (AN, Agua Nueva Water Reclamation facility, Tucson), anaerobic sludge obtained from different full-scale bioreactor systems treating recycled paper wastewater (sludges R and E), and excess activated sludge (INA, Ina wastewater treatment plant, Tucson).

Table 3.1. Volatile suspended solids (VSS) content in the sludges used in this study.

Sludges	Volatile suspended solids (VSS) per unit wet weight
Mahou	6.57 ± 0.85 g VSS/g
INA	2.19 ± 0.009 g VSS/L
Rockteen	0.096 ± 0.0012 g VSS/g
Eerbeek	0.11 ± 0.0026 g VSS/g
Agua Caliente	0.031 ± 0.0023 g VSS/g
Agua Nueva	0.034 ± 0.0011 g VSS/g

3.2.2. Basal medium

Different media were used in the toxicity and reduction tests, respectively. The composition of the medium used in the toxicity tests (M1) is provided in Table 3.2. The medium M1 contained acetate as a source of carbon and as electron donor and all the trace elements needed by the microorganisms (Table 3.3). The composition of the medium used in the Sb (V) bioreduction experiments is listed in Table 3.4. The composition of the trace elements solution is shown in Table 3.3. In addition, the medium was supplemented with an electron donating substrate. A low-chloride medium (M2) was used in microbial reduction bioassays (Table 3.4) to minimize interference by chloride during the analysis of Sb (V) using ion chromatography. This medium lacked acetate because hydrogen was used as electron donor (8 psi).

Table 3.2. Basal mineral medium used for acetoclastic methanogenic toxicity assays (M1)

Compounds	mg/L
Total Volume	1 L
CH_3COONa	2563*
K_2HPO_4	250
$CaCl_2.2H_2O$	10
$MgSO_4.7H_2O$	100
$MgCl_2.6H_2O$	100
NH_4Cl	280
$NaHCO_3$	4000
Yeast Extract	100
Trace element solution	1

*2563 mg/L yields 2 g COD/L (0.78 g COD/g CH_3COONa).

This amount of CH_3COONa yields a theoretical maximum of 12.6% CH_4 (v/v)

Table 3.3. Composition of the trace element solution used in all the anaerobic bioassays.

Compound	mg/L
H_3BO_3	50
$FeCl_2 \cdot 4\ H_2O$	2000
$ZnCl_2$	50
$MnCl_2 \cdot 4H_2O$	50
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	50
$AlCl_3 \cdot 6\ H_2O$	90
$CoCl_2 \cdot 6\ H_2O$	2000
$NiCl_2 \cdot 6\ H_2O$	50
$CuCl_2 \cdot 2\ H_2O$	30
$NaSeO_3 \cdot 5\ H_2O$	100
EDTA	1000
Resazurin	200
36% HCl	1 mL

Table 3.4. Basal mineral medium used for Sb (V) microbial reduction assays (M2)

Compounds	mg/L
Total Volume	**1 L**
K_2HPO_4	250
$CaCl_2.2H_2O$	10
$MgCl_2.6H_2O$	10
NH_4HCO_3	206.9
$NaHCO_3$	3780.1
Yeast Extract	100
Trace element solution	1

The medium used in the Sb (V) reduction experiments was modified further in an attempt to improve the separation of Sb (V) by ion chromatography. While working with the IC analyzing samples from the reduction tests we found that some peaks were overlapping with the Sb (V) peak complicating the integration. The composition of the modified medium (M3) is listed in Table 3.5.

Table 3.5. Modified basal medium used for Sb (V) microbial reduction assays (M3)

Compounds	mg/L
Total Volume	**1 L**
K_2HPO_4	12.5
$CaCl_2.2H_2O$	10
$MgCl_2.6H_2O$	10
NH_4HCO_3	20
$NaHCO_3$	2000
Yeast extract	10
Trace element solution	0.2

3.2.3. Methanogenic toxicity tests

Batch experiments were conducted in duplicate using glass serum flasks (160 mL) containing 50 mL of basal medium M1 (Table 3.2.) supplemented with Mahou sludge (1.5 g VSS/L) and acetate (2.56 g/L). The flasks were sealed with butyl rubber stoppers and aluminum crimp seals. Next, the headspace was flushed with a mixture of N_2 and CO_2 (80:20, v/v) to create anaerobic conditions. All flasks were pre-incubated overnight in an orbital shaker (115 rpm) at 30±2°C to ensure that the methanogens were adapted to the experimental conditions. The following day, a certain quantity of Sb (V) or Sb (III) was added depending on the desired concentration. Controls lacking toxicant addition and controls without sludge were run in parallel. Subsequently, the flasks were incubated in an orbital shaker (115 rpm) at 30±2°C. Methane (CH_4) production was determined periodically until 80% or more of the substrate in toxicant-free controls was depleted.

The normalized microbial activity (NMA) was calculated as shown below:

$$NMA\ (\%) = \frac{Maximum\ Specific\ Activity\ of\ Experimental\ Group}{Maximum\ Specific\ Activity\ of\ Control}\ x\ 100$$

The maximum specific activity was calculated from the slope of cumulative CH_4 production.

The measurement of the methane was performed using a gas chromatograph (GC). Sampling was made twice every day by injecting gas from the headspaces. Every sample was injected three times to minimize the experimental errors.

3.2.4. Microtox toxicity test

The Sb toxicity was tested using a Microtox standardized test. Microtox is an in vitro, metabolic inhibition test system that uses a strain of a naturally bioluminescent marine bacterium named

Aliivibrio fischeri that produces light as byproduct of cellular respiration. The toxicity of test chemicals can be recognized by the loss of luminescence level that results from cellular activity inhibition.

In the study, both species (Sb (V) and Sb (III)) were tested using a Microtox M500 analyzer (Waters, Milford, MA, USA) following the Microtox acute toxicity test protocol (EPA. 1999). Microtox reagent (*A. fischeri*), reconstitution solution (ultra-pure water), osmotic adjusting solution (22% NaCl), and diluent (2% NaCl) were obtained from Fisher Scientific (Hampton, NH, USA). Stock solutions of Sb (III) and Sb (V) (500 mg/L) were prepared prior to the experiments. Then, nine dilutions were prepared by a 2-fold serial dilution using Microtox diluent. The concentration ranges used in this assay were: 1.95–500 mg/L for Sb (III) and Sb (V). Luminescent levels were tested after 0, 15 and 30 min of exposure. All tests were performed in duplicate; blank controls and samples with citrate alone were run in parallel. Percent microbial activity was calculated.

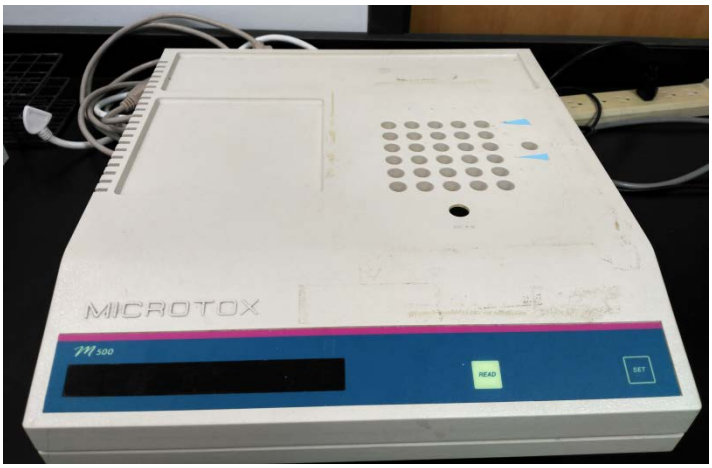

Figure 3.1. *Analyzer used for the Microtox bioassay.*

In the Microtox test, the parameter gamma (Γt), the radio of the light loss to the remaining light level at time t is commonly used for data processing. The value of light loss is achieved by the following equations (U.S. EPA, 2003):

$$\Gamma_t = \frac{\text{Corrected Light Loss}}{\text{Remaining Light Level}} = \frac{R_t \cdot I_0 - I_t}{I_t} = \frac{R_t \cdot I_0}{I_t} - 1 \qquad (1)$$

$$R_t = \frac{C_t}{C_0} \qquad (2)$$

Where: R_t is the light output ratio of negative control at time t (Ct) to the value at time 0 (C0). It is used to correct and normalize the light variation in the test. I0 and It are the light levels of the samples at time 0 and time t. Percent inhibition and activity can be derived by the following equations:

$$\text{Inhibition}(\%) = \frac{\text{Light Loss}}{\text{Light Loss+Ramaining light}} = \frac{\Gamma_t \cdot I_t}{\Gamma_t \cdot I_t + I_t} \times 100 = \frac{\Gamma_t}{\Gamma_t + 1} \times 100 \qquad (3)$$

$$\text{Activity}\ (\%) = 100 - \text{Inhibition}(\%) = \left(1 - \frac{\Gamma_t}{\Gamma_t + 1}\right) \times 100 = \frac{1}{\Gamma_t + 1} \times 100 \qquad (4)$$

3.2.5. Microbial reduction of Sb (V)

These tests were performed in 160 mL serum flasks supplied with 100 mL of basal medium M3 (Table 3.5) supplemented with 1.5 g VSS/L of the different inoculum tested (Table 3.1). The liquid phase was flushed with a mixture of N_2 and CO_2 (80:20, v/v). Then, the flasks were sealed with butyl rubber stoppers and aluminum crimp seals. Next, the headspace was flushed with a mixture of N_2 and CO_2 (80:20, v/v) to create anaerobic conditions. After that, hydrogen was added (8 psi,

about x30 of the stoichiometric pressure needed) in some bottles as electron donor. 20 mg/L of Sb (V) were added after overnight preincubation. Controls lacking toxicant addition and controls without sludge were run in parallel. The flasks were incubated in an orbital shaker (115 rpm) at 30±2°C.

Samples of the liquid medium (approx. 1.5 mL) were obtained periodically to evaluate the change in the soluble Sb concentration and Sb speciation. The sampling time was different depending on the inoculum used on each test. All the samples were centrifuged for 10 min at 12,000 rpm to remove suspended solids from the liquid.

3.3. ANALYTICAL METHODS

3.3.1. Ion chromatography

The concentration of soluble Sb (V) was analyzed by ion chromatography (IC). The IC system employed in this work was a Dionex Integrion HPIC system (Sunnyvale, CA, USA) with a Dionex Ion-Pac AS18 analytical column (4 × 250 mm; Thermo Scientific, Waltham, MA, USA) and an IonPac AG18 guard column (4 × 50 mm). The IC system was equipped with an integrated eluent (potassium hydroxide) generator using a 10 mM eluent at a flow rate of 1 mL/min. The chromatograms were acquired using Chromeleon software, version 7, also supplied by Dionex. The retention time for Sb (V) was about 3.7 min. Sb (III) is not retained by this column. A sample volume of 200 µL was injected in all the measurements. The Sb (V) detection limit was 1 mg/L. The calibration curve used contained the following concentrations; 1, 5, 10, 25, 50 and 100 mg/L.

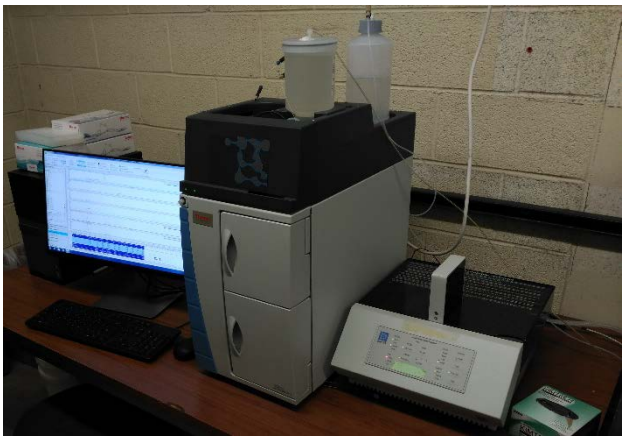

Figure 3.2. Ion chromatograph used to analyze the samples.

The samples must have a pH of about 7. The samples were diluted to ensure that the Sb (V) concentration was less than 100 mg/L because of the calibration curve.

3.3.2. Total antimony (ICP-OES)

The soluble concentration of Sb in liquid samples was measured using an inductively coupled plasma–optical emission spectrometry (ICP–OES) system (Agilent Technologies 5100, Santa Clara, CA, USA). The wavelengths used were 206.834 and 217.582 nm, noticing that the second one provides better results. All the samples were diluted in nitric acid 2% (v/v) and the eluent used to rinse the autosampler was also nitric acid 2%. The instrument was calibrated before analysis using Sb standards ranging in concentration from 50 µg/L to 5 mg/L. The detection limit was 50 µg/L.

3.3.3. Antimony speciation (HPLC-ICP-OES)

A method developed in our laboratory was used to determine the soluble concentration of Sb (V) and Sb (III) in liquid samples. An HPLC pump (Waters, Millipore 590, Milford, MA, USA) fitted with an chromatographic column (Dionex AS15 4 x 250 mm) and a guard column (Dionex AG15 4 x 50 mm), both from Thermo Scientific, was coupled to an ICP-OES (Agilent Technologies 5100, Santa Clara, CA, USA) in the continuous measurement mode. The eluent used was ethilendiaminetetraacetic acid (EDTA) 40 mM at a flow rate of 1 mL/min. To inject the samples we used a 300 µL loop. To ensure a good determination of antimony species we used two wavelengths; 206.834 and 217.582 nm. The detection limit for this method was 50 µg/L for Sb (V) and 100 µg/L for Sb (III). A simple scheme is shown below in Figure 3.3.

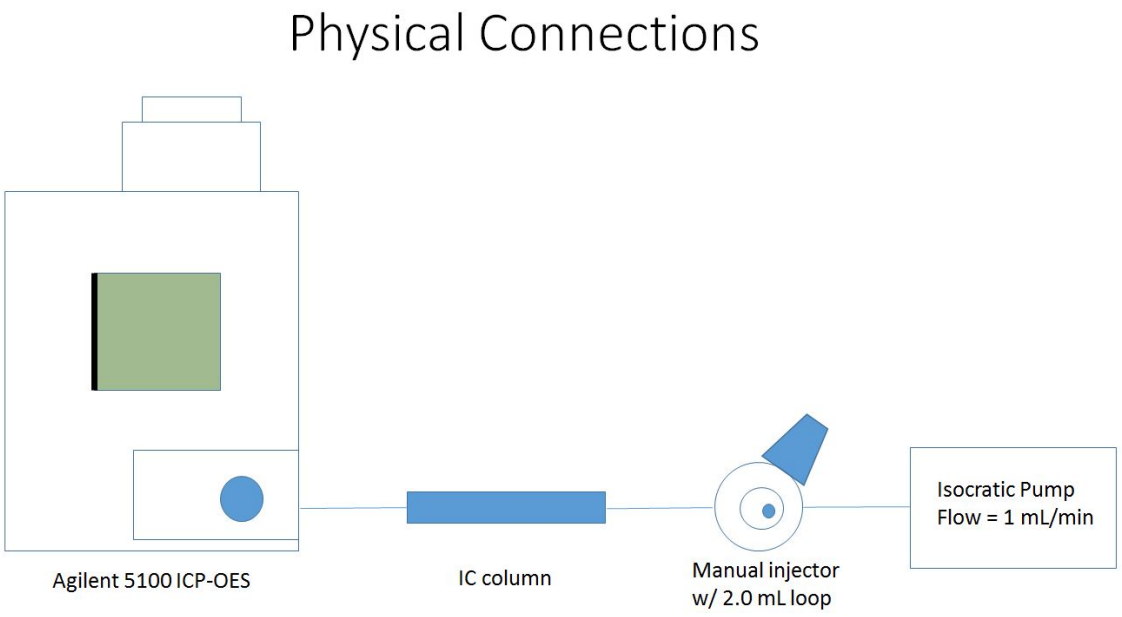

Figure 3.3. *Scheme showing the connections used in the HPLC-ICP-OES instrument used for Sb speciation.*

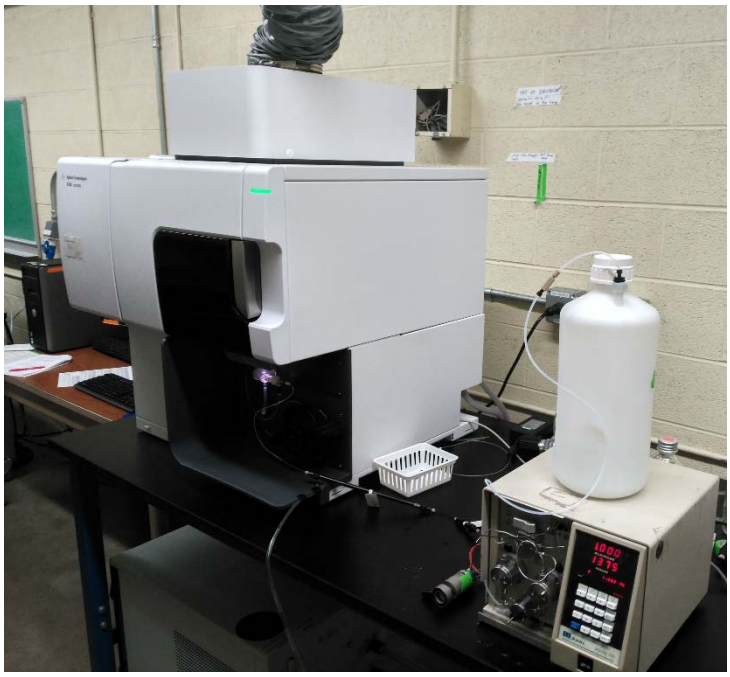

Figure 3.4. *HPLC-ICP-OES instrumentation used for Sb speciation.*

There are several conditions we need in order to make the method work. The image below shows different conditions used and the reason of its values depending on what we want.

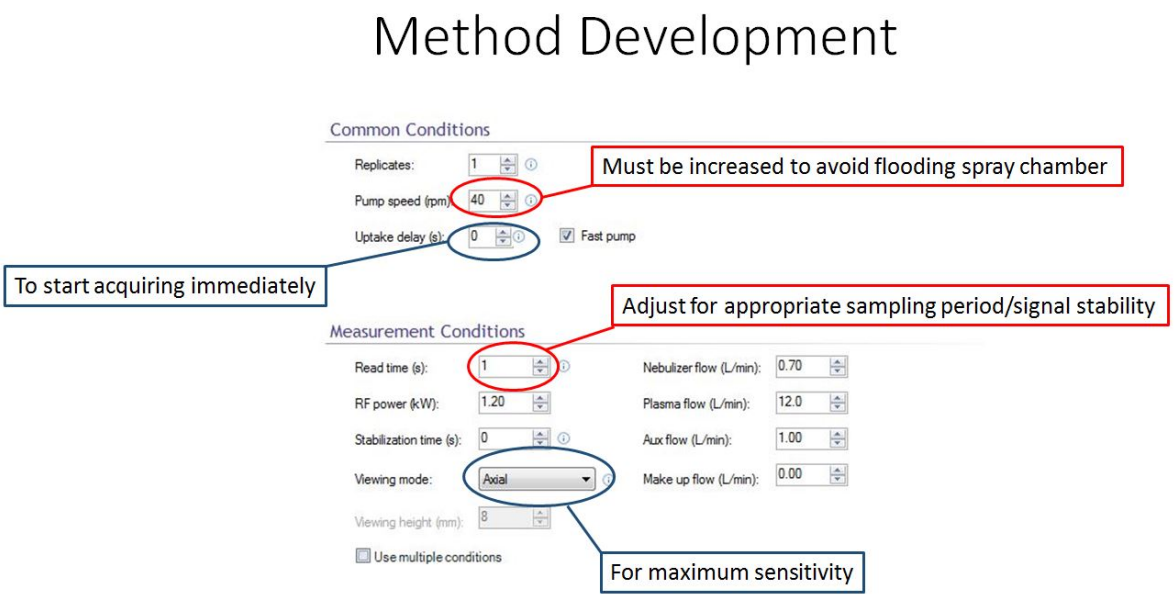

Figure 3.5. *ICP-OES configuration used in the speciation method.*

The synchronization between the HPLC and the ICP-OES runs is done manually:

- The sample is injected in the loop (in load position).
- Now we change the injector to the inject position.
- The ICP-OES data collection is manually started.

The second and third steps should be done at the same time. This needs two people to correctly synchronize it.

The data collected was exported to Excel as a series of numbers. I developed an Excel method to integrate these peaks obtained in the chromatogram by just introducing approximate times before and after each peak. Using this program it is possible to integrate at the same time two different wavelengths and, for each wavelength, four different peaks.

3.3.4. Gas chromatography

The CH_4 content in headspace samples were measured using a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with He as the carrier gas. CH_4 was determined using a flame ionization detector and a Restek Stabilwax-DA column (30 $\times$ 0.35 mm, ID 0.25 µm) (Karri et al. 2005).

Every time the analysis was run seven standards were measured with 0.49, 1.49, 1.99, 4.98, 9.97, 14.97 and 19.95 % v/v of methane. The measurements were made three times for every

bottle to work with the media and minimize the error. The image below shows a calibration curve obtained in one of those runs.

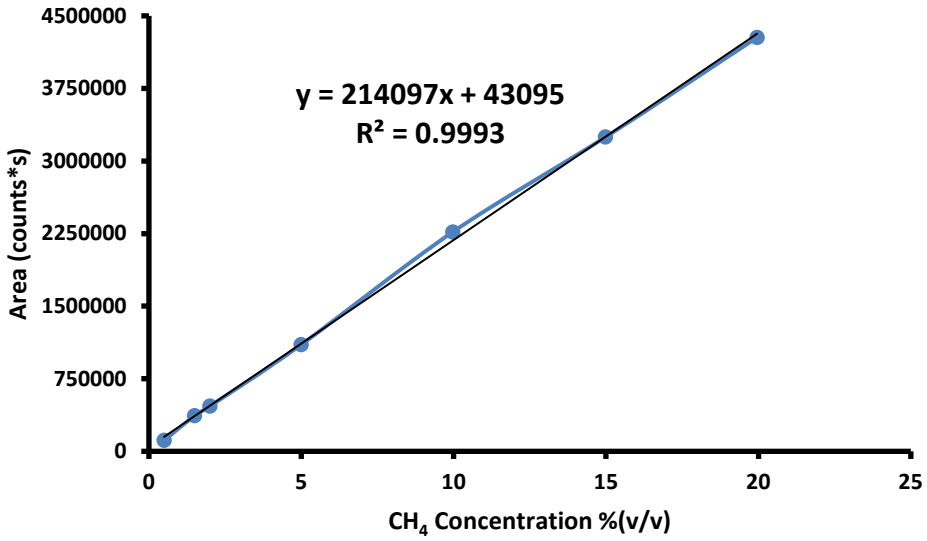

Figure 3.6. Methane calibration curve for the GC.

3.3.5. pH and other determinations

The concentration of total suspended solids (TSS) and volatile suspended solids (VSS) in the various inocula was measured according to Standard Methods (Clesceri LS et al. 2013).

4. RESULTS & DISCUSSION

4.1. Development of analytical methods for Sb speciation

4.1.1. Ion chromatography (IC) with suppressed conductivity detection

Sb (V) is ionized at the circumneutral pH values typical of our bioassays (Filella M. et al. 2002b). Therefore, ion chromatography might be a suitable method to quantify Sb (V). On the other hand, Sb (III) is not ionized over a broad pH range (2-10) (Filella M. et al. 2002b) and, as a consequence, it can not be detected using a conductivity detector. Nonetheless, Sb (III) could be determined indirectly by subtracting the Sb (V) concentration from the total Sb concentration in the samples. This would be helpful as speciation of Sb (III) and Sb (V) is very time consuming.

In the initial phase, several runs were performed to optimize the method, figure out what the retention times were for each component, if there were any important interferences, and check if the results were repetitive. At first we tried measuring Sb (V) to identify its retention time (about 3.7 to 4.0 min with the final configuration).

The image below shows three chromatograms comparing; (a) medium, (b) 100 mg/L of Sb (V) and (c) medium containing 100 mg/L of Sb (V) (Figure 4.1). These samples were analyzed using 20 mM KOH as eluent. However, under these conditions the different peaks were overlapping, indicating the need for a method modification.

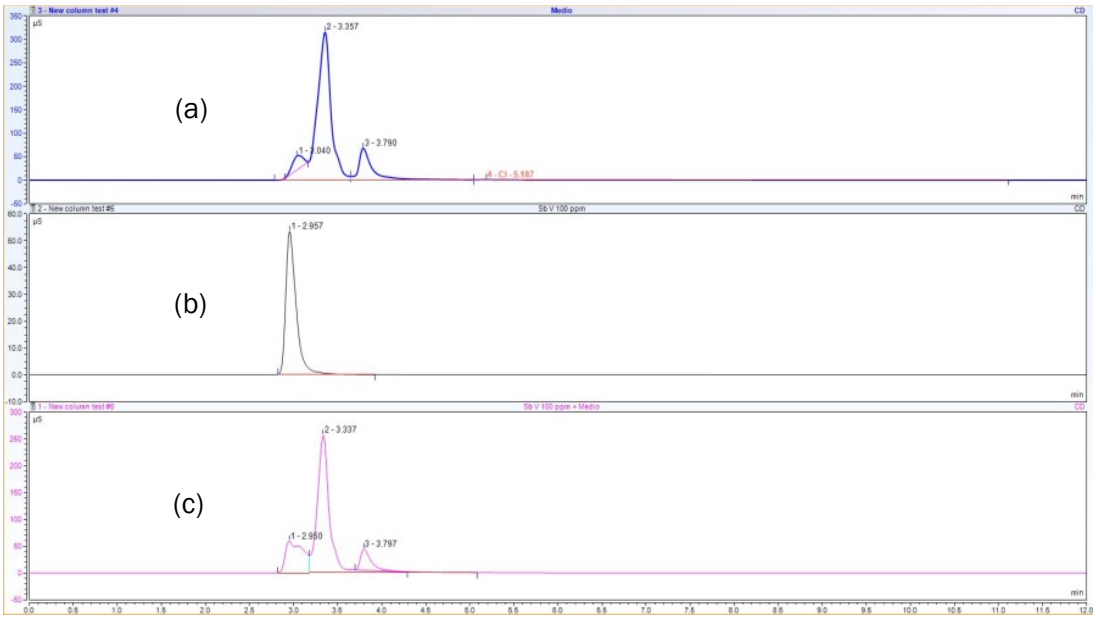

Figure 4.1. *First chromatograms obtained (before method optimization); (a) shows a sample containing clean medium, (b) demineralized water with 100 mg/L of Sb (V,) and (c) medium with 100 mg/L of Sb (V).*

To reduce as much as possible all the interferences, the amount of chloride in the medium was reduced (Table 3.4) and different eluent concentrations (10-40 mM KOH) were tested. Similarly to Sb (V), chloride elutes at the beginning of the run (about 4 min retention time). The best separation was attained using 10 mM KOH (results not shown). Using this eluent, a run time of 10 min was sufficient to elute the peaks in the sample (even though we always used more to prevent any compound remaining and interfering with the next chromatogram).

An additional problem was the low sensitivity of the conductivity detector for antimony. To minimize this problem, the injection loop was replaced in order to increase the injection volume from 25 to 200 µL. Along with the intensity of the antimony peak, the intensity of other peaks also increased resulting in some overlap between the antimony and the chloride peak. Therefore, the basal

medium was modified again to reduce the concentration of chloride and minimize these interferences (Table 3.5).

Sb (V) calibration samples were prepared and analyzed in duplicate to evaluate the repeatability and linearity of the IC analysis. Figure 4.2 shows the chromatograms obtained for the Sb (V) calibration samples. The concentrations used were 1, 5, 25, and 50 mg/L and all of them were duplicated. The response was linear (R^2 = 0.9993) and the standard deviations were really low (they are represented in the next figure but are smaller than the markers).

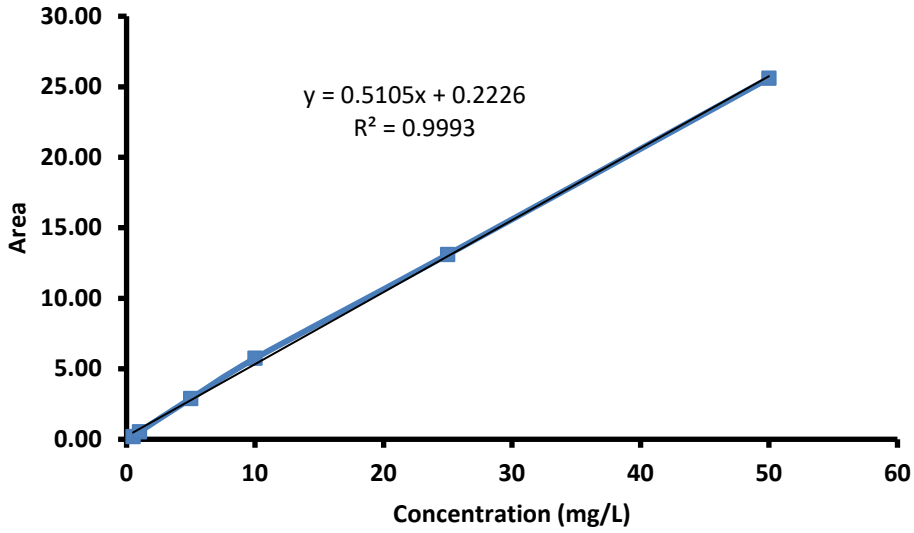

Figure 4.2. Sb (V) calibration curve with standard deviations.

Figure 4.3 shows the chromatograms obtained for Sb (V) calibration samples prepared in the medium used for the reduction tests (Medium M3, Table 3.5). Unfortunately, some components in the medium seem to interfere with the detection of Sb (V), When the Sb (V) concentration increased, the peak became wider and smaller. The relation area-concentration was still linear so even with these strange peaks the analysis could still work measuring the samples to quantify the amount of soluble Sb (V). Analysis of Sb (V) samples incubated with Agua Caliente inoculum in medium M3 for 0, 24 and 48 h under reducing conditions indicated that the detection of Sb (V) was further complicated due to the release of interfering compounds from the inoculum (Figure 4.4). Based on these results, we concluded that detection of Sb (V) by IC is unviable under the conditions of our reduction experiments.

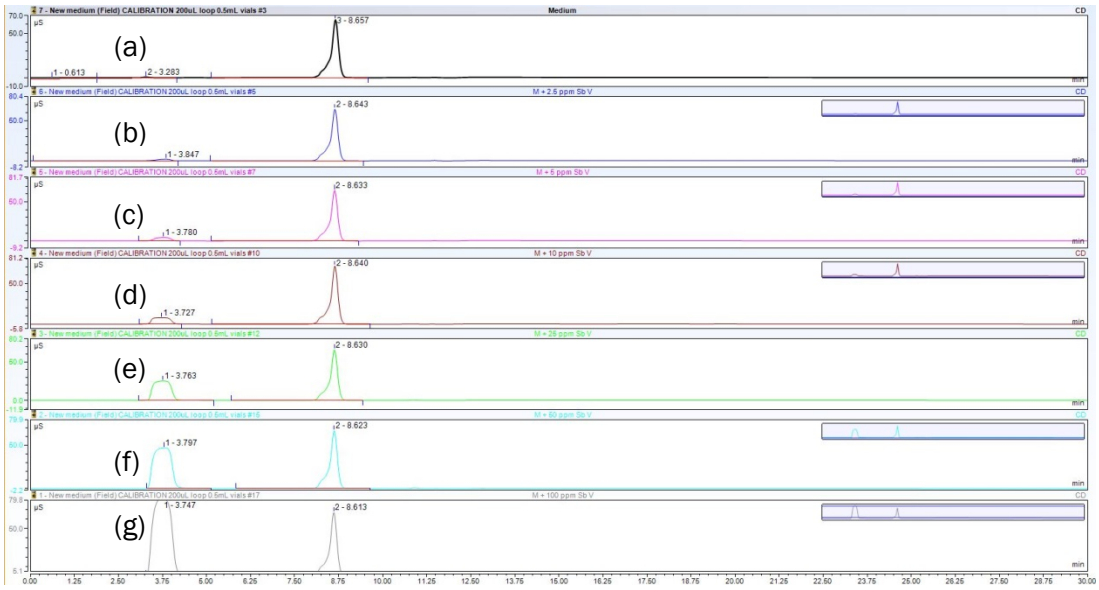

Figure 4.3. *Sb (V) calibration curve with medium, 10 mM eluent concentration and 30 min run. Concentrations of Sb (V) (in mg/L): (a) 0, (b) 2.5, (c) 5, (d) 10, (e) 25; (f) 50, and (g) 100.*

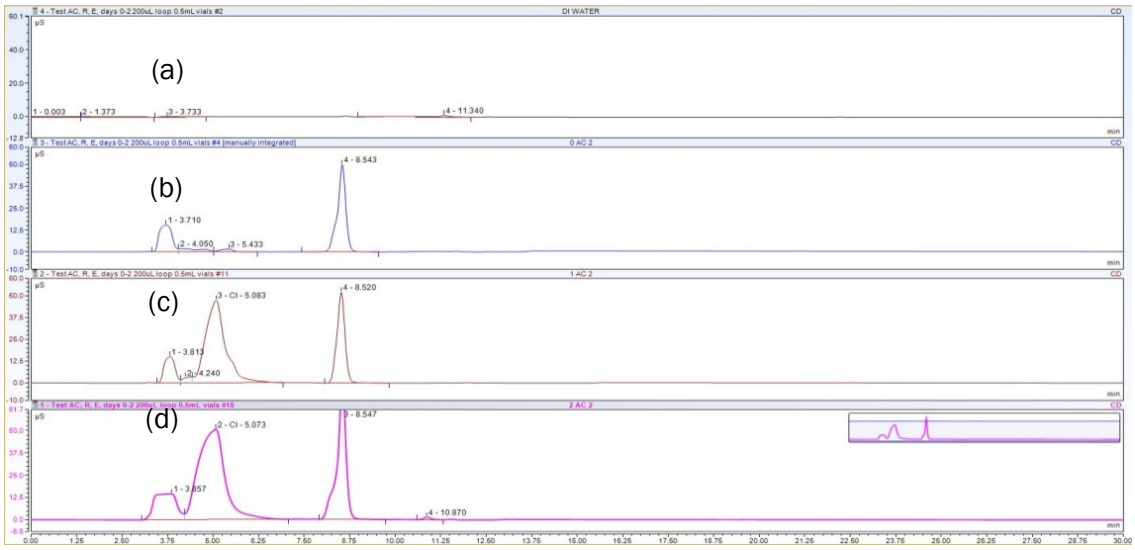

Figure 4.4. *"Agua Caliente" samples (times 0, 24, 48 h) analysis and the interference found. (a) Deionized water used to dilute the samples, (b) sampling time 0 h, (c) sampling time 24 h and (d) sampling time 48 h.*

4.1.2. ICP-OES

ICP-OES analysis was used to determine the concentration of total soluble antimony in liquid samples. The method worked without any problem and it is very reliable. Figure 4.5 shows the calibration curves determined for Sb at two different wavelengths (206.834 and 217.582 nm). Both curves are very good (R^2 = 0.9996) but we noticed that the second wavelength (217.582 nm) had a better performance because of its resolution and this allows us to measure more precisely the samples. The detection limit was 50 µg/L of antimony (The ICP-OES does not distinguish between Sb (V) and Sb (III)). The calibration curve is shown in the graph below.

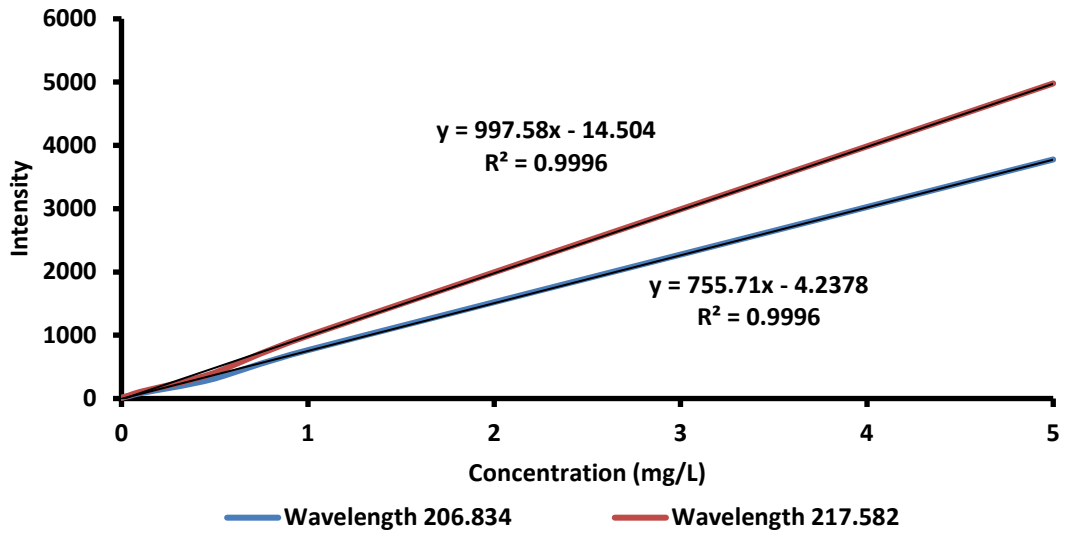

Figure 4.5. *Calibration curves determined for total antimony in the ICP-OES using different wavelengths.*

Depending on the wavelength used there are some different interferences that can affect the measurements. The possible interferences for both these wavelengths are shown in the next two tables showing the elements, the intensity returned by these elements and the exact wavelength for them.

Table 4.1. *Possible interferences for the first wavelength (206.834 nm) used in the speciation analysis.* The only wavelengths considered as possible interferences are the ones above 100 intensity value and with not more than 0.2 nm difference with the wavelength used.

Element	λ	Intensity
Sb (Used)	206,834	1316,3
Pt	206,75	113,1
W	206,752	190,3
Ge	206,866	903,9

Table 4.2. Possible interferences for the second wavelength (217.582 nm) used in the speciation analysis. The only wavelengths considered as possible interferences are the ones above 100 intensity value and with not more than 0.2 nm difference with the wavelength used.

Element	λ	Intensity
Sb (Used)	217,582	1973,8
Ni	218,467	3940,4
Pt	217,468	1334,8
Be	217,51	1710,6
Ni	217,514	2439,4
Ir	217,525	147,5
Hf	217,535	525
Nb	217,584	288,3
Re	217,62	268,5
Nb	217,676	670,3

4.1.3. Speciation

Usually the method used to speciate metals/metalloids is the HPLC-ICP-MS (Jabłońska-Czapla et al. 2014, Krachler Michael and Hendrik 2001, Müller et al. 2009, Nina 1998). This instrumentation is very sophisticated and expensive so there are only a few of these instruments available on the campus of the University of Arizona. ICP-OES can be used to measure metal concentrations and these instruments are widely available. That is why the decision to develop a method using HPLC-ICP-OES was made.

This method played an essential role during all the research performed. Data is obtained as a series of numbers for each wavelength used and all the results have to be calculated from them. At first this took a lot of time for each sample analyzed so an excel worksheet was developed to make it as fast and easy as possible to save lots of time in the data processing. All the design

process, the different filters applied and how the final results are shown for the excel worksheet is explained in detail in the appendix section (APPENDIX 2: Integration Excel worksheet).

The first thing needed was to optimize the method in order to obtain good peaks, a good separation and enough points in each peak (about 10 to 15 points) in order to have a good definition of them. Different eluent (EDTA) concentrations were tried and finally it was decided to use 40 mM (15 mg/L) because the peaks were quite good defined and the retention times were different enough (about 2 min lapse between the first and the second peak). The first peak represents Sb (V) (3 min retention time) and the second one represents Sb (III) (5 min retention time). Sb (V) has a higher analysis sensibility returning a higher intensity value for the same amount injected. Results obtained using 40 mM eluent concentration and two different wavelengths are shown below.

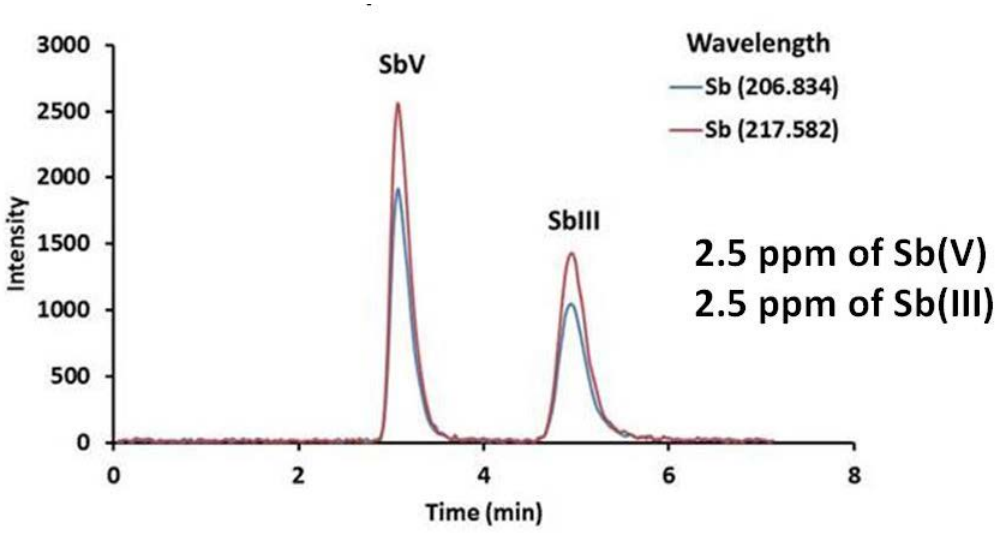

Figure 4.6. *Chromatogram obtained from the HPLC-ICP-OES speciation method (5 mg/L total Sb injected).*

Another thing needed in order to trust the results obtained with this method were the calibration curves for both antimony species. The concentrations used were 12.5, 25, 50, 125, 250, 500, 1000, 2500 and 5000 µg/L in order to determine the limit of detection for both species. In addition, three different wavelengths were used to look for the best one while analyzing antimony. Every time an analysis was run, at least two different wavelengths were used to make sure that, if the chromatogram returns a peak, it is antimony and not any interference. The interferences are the same than in the ICP-OES excepting for the extra wavelength tested for this method (231.146 nm). The table below shows the possible interferences for this wavelength and their magnitude.

Table 4.3. *Possible interferences for the third wavelength (231.146 nm) used in the HPLC-ICP-OES speciation analysis.*

Element	λ	Intensity
Sb (Used)	231,146	1843
Pt	231,095	453,5
Ni	231,096	4900
Co	231,16	13310,5
Ni	231,234	2551,9
Co	231,256	310,8
Ta	231,261	865

The next two figures show the calibration curves obtained for Sb (V) and Sb (III). Standard deviations are shown, but sometimes there are not visible because of its low value.

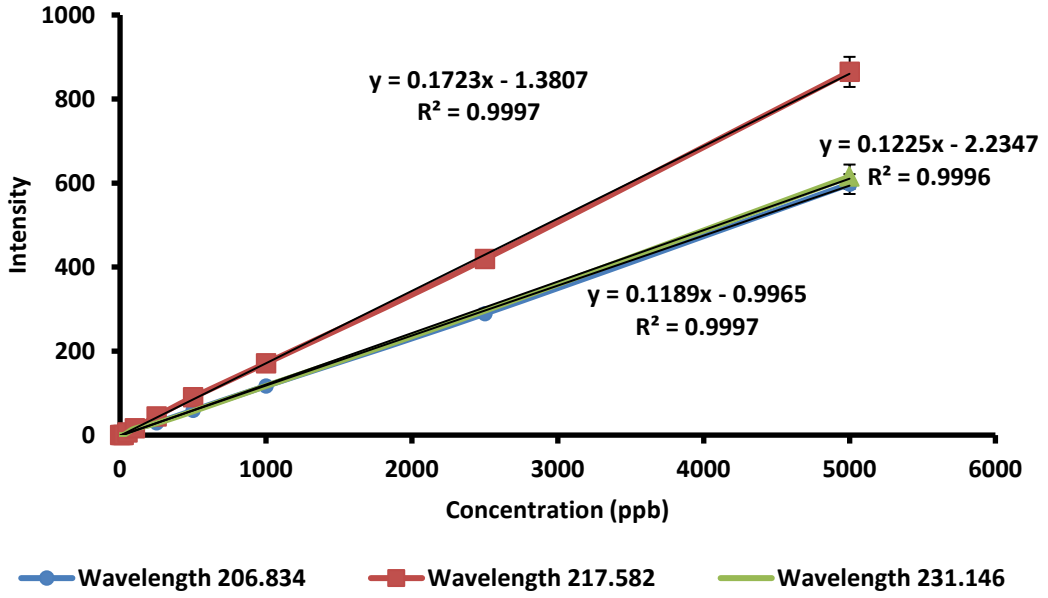

Figure 4.7. Sb (V) calibration curve obtained with the HPLC-ICP-OES speciation method.

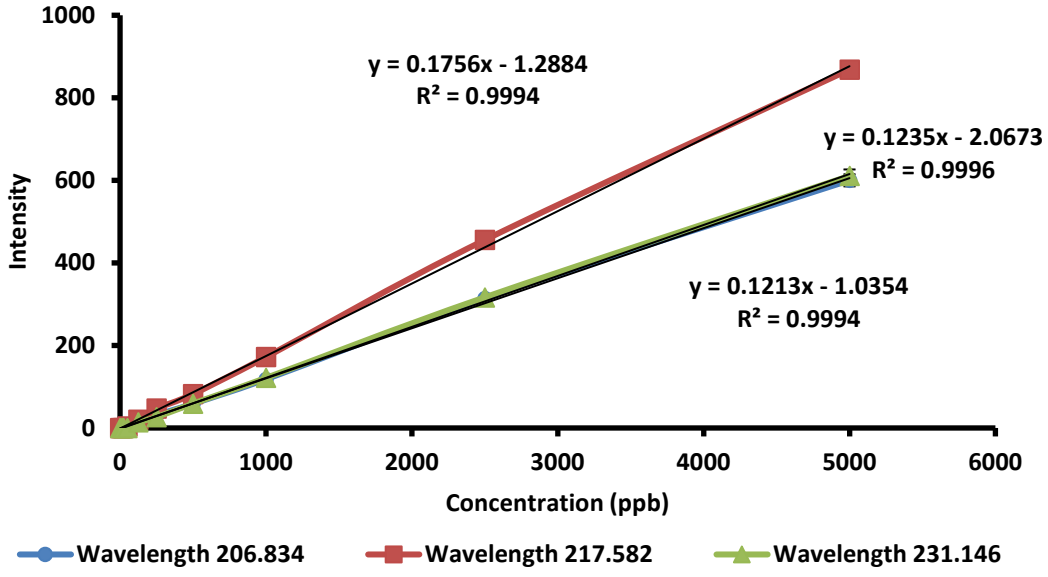

Figure 4.8. Sb (III) calibration curve obtained with the HPLC-ICP-OES speciation method.

4.2. Microbial toxicity

4.2.1. Microtox

The Microtox bioassay showed that Sb (V) is not toxic at relatively high concentrations (up to 500 mg/L) after 30 min of exposure time. Measurements were taken at 5, 15 and 30 min and the activity remained constant. On the other hand Sb (III) showed a high toxicity with the same exposure times. Sb (III) caused 50% inhibition at 100 mg/L after only 15 min exposure and about 60 mg/L after 30 min. These results are shown in the next two figures, respectively.

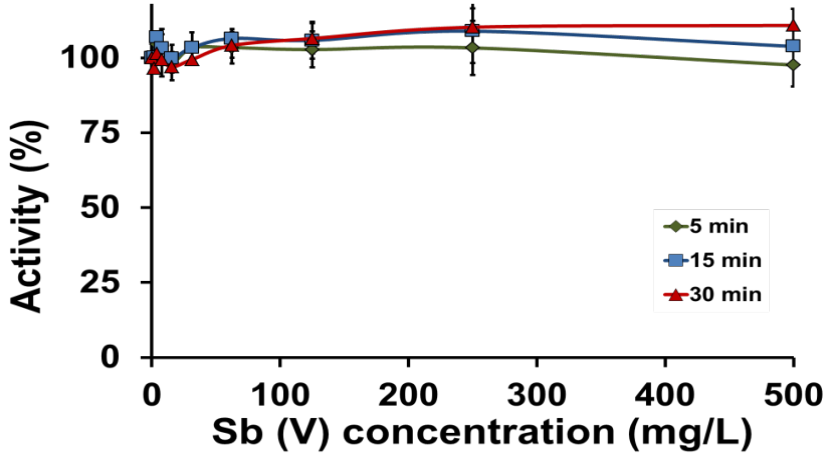

Figure 4.9. Sb (V) Microtox test.

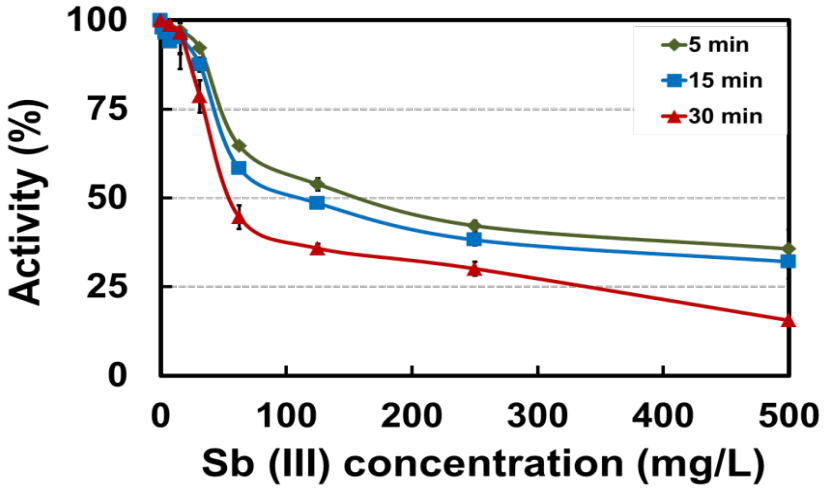

Figure 4.10. Sb (III) Microtox test.

4.2.2. Anaerobic methanogenic toxicity

The first experiment run consisted in bottles with different concentrations of Sb (V) (50, 100, 150, 200 and 250 mg/L) and Sb (III) (5, 10, 25, 50, 100 mg/L). Figures 4.11.and 4.12 illustrate the time course of methane production as a function of the concentration of Sb (V) and Sb (III), respectively. Later some more concentrations of Sb (III) were added (12, 15, 18, 21 mg/L) when the gap between 10 and 25 mg/L was visible to try to make the graph more precise. The final results obtained after all the concentrations tested are shown in Figures 4.11 and 4.12. The toxicity difference between both species can be easily appreciated. As expected, Sb (III) is more toxic that Sb (V).

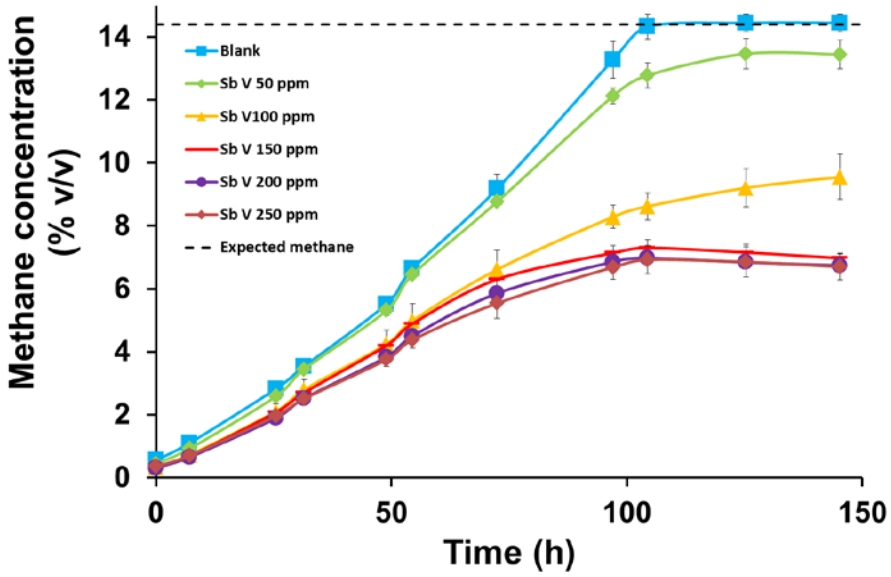

Figure 4.11. *Methane production as a function of incubation time in hydrogenotrophic methanogenic toxicity bioassays inoculated with "Mahou" sludge and exposed to increasing concentrations of Sb (V).*

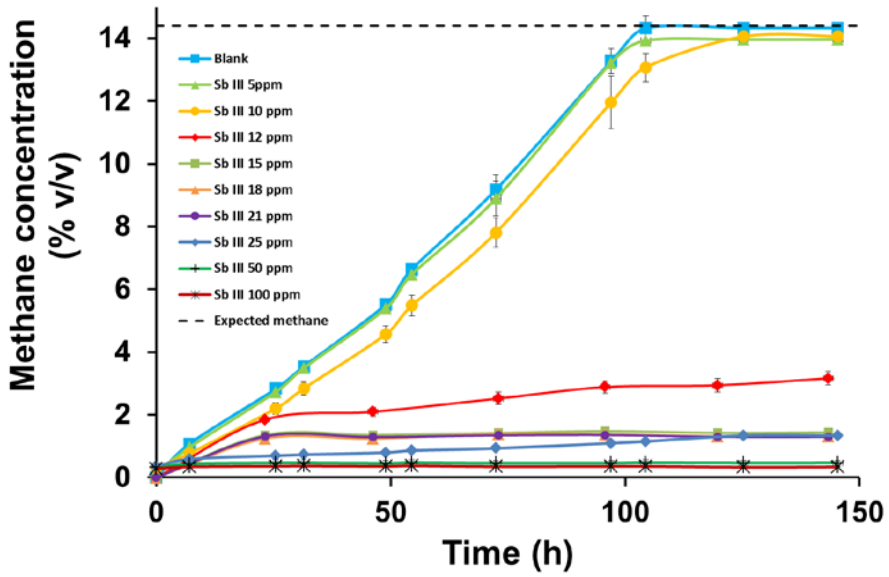

Figure 4.12. *Methane production as a function of incubation time in hydrogenotrophic methanogenic toxicity bioassays inoculated with "Mahou" sludge and exposed to increasing concentrations of Sb (III).*

The normalized methanogenic inhibition (as percentage of the control) as a function of the concentration of Sb (V) and Sb (III) is shown in Figures 4.13. The 50% inhibiting concentration (IC50) was found as 150 mg/L for Sb (V) and between 10 and 12 mg/L for Sb (III).

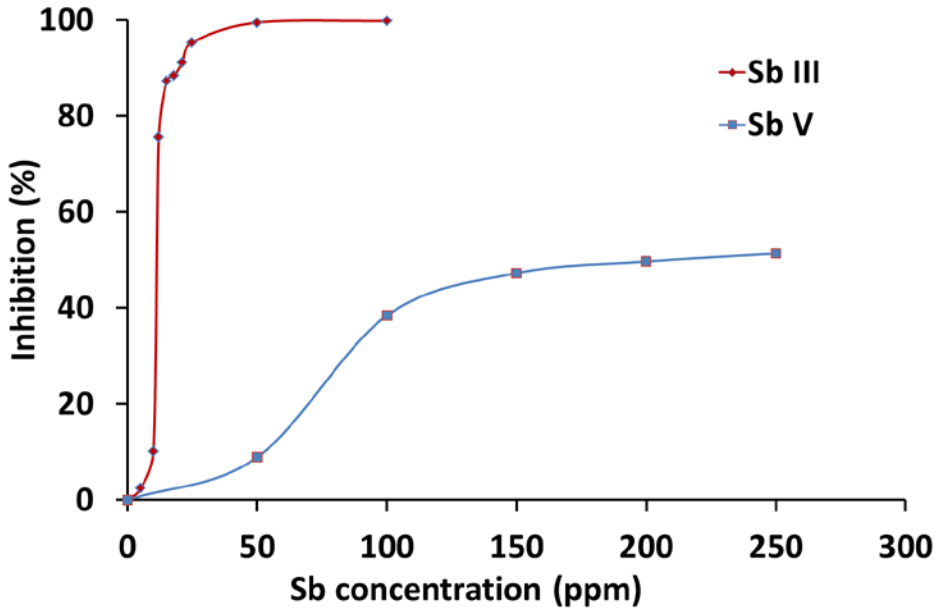

Figure 4.13. *Normalized methanogenic inhibition (%) as a function of the concentration of Sb (III) and Sb (V) used.*

To investigate the impact of exposure time on the inhibitory potential of Sb (V), the medium in the bioassay described above was replaced with a new one containing the same Sb (V) concentration as the previous one and the assay was incubated again. The results obtained from the second feed were quite interesting. Sb (V) seemed to completely inhibit the methane production at concentrations of 150 mg/L or higher, so Sb (V) showed to be much more toxic at a long exposure time than at a shorter one but it needs quite high concentrations (4.14). The normalized methanogenic inhibition (as percentage of the control) as a function of the concentration of Sb (V) for the second feed is shown in Figure 4.15.

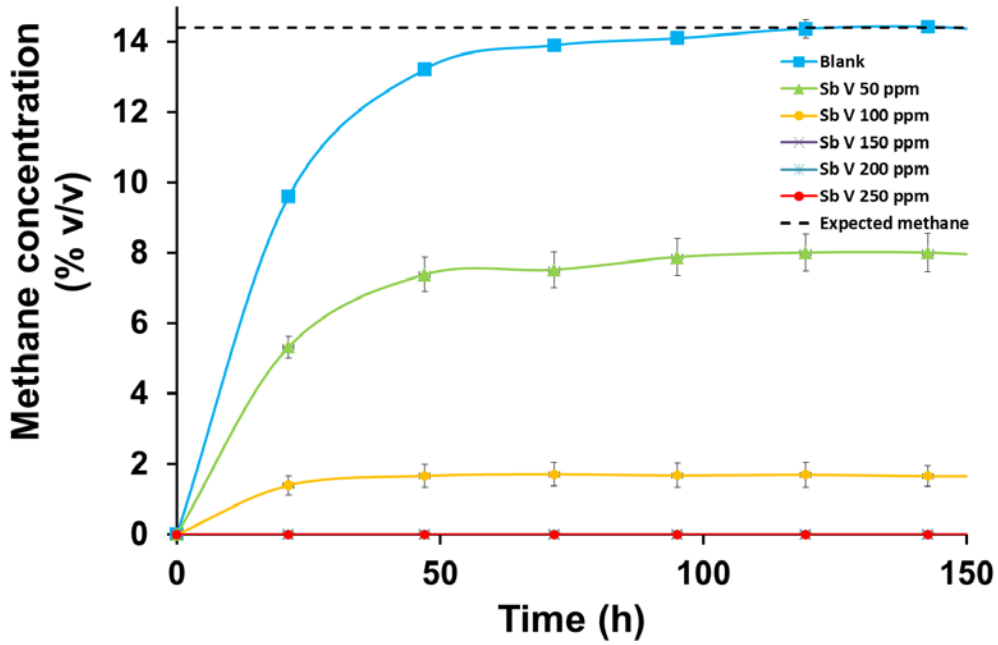

Figure 4.14. *Time course of methane production as a function of the Sb (V) concentration in the second feeding.*

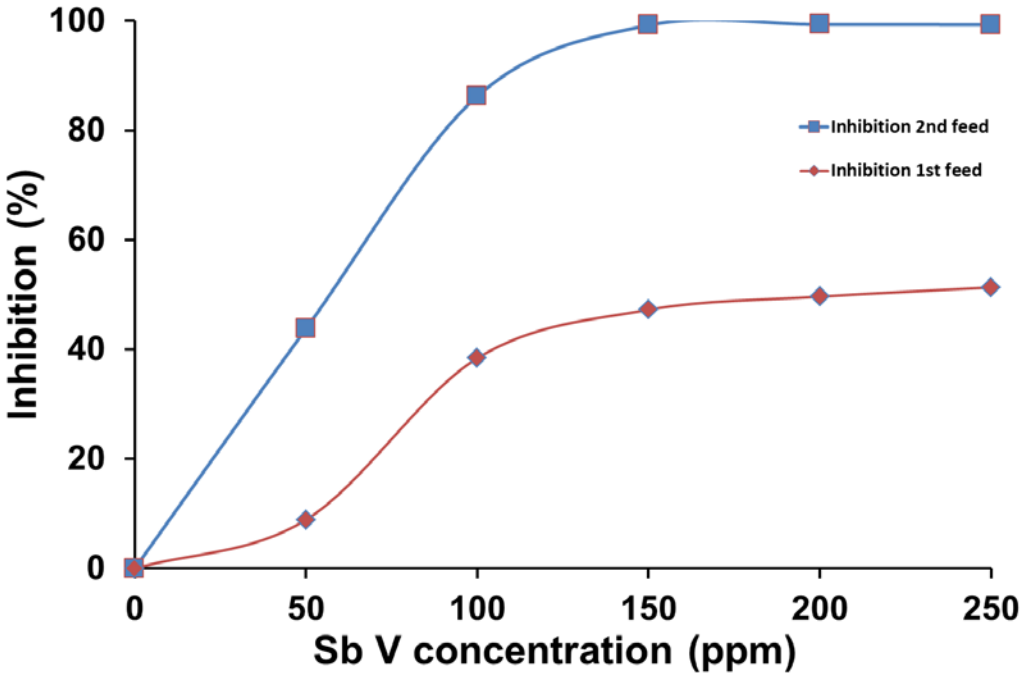

Figure 4.15. *Normalized methanogenic inhibition (%) comparing the first and second feeding obtained as a function of the concentration of Sb (V) used.*

The IC50 value obtained for this second feed is slightly higher than 50 mg/L. This value is three times lower than the one obtained with the first feed and it is not a really high concentration. This means that in a long exposure time Sb (V) is definitely more toxic than in a short one.

4.3. Bioreduction of Sb (V) under anaerobic conditions

4.3.1. Visual experiments

Screening tests were set up to obtain visual evidence for the reduction of Sb (V) to Sb (III). A high concentration of Sb (V) (250 mg/L) in medium M3 (Table 3.5) was used to facilitate the detection of reduced Sb. Sb (III) is poorly soluble in water (max. solubility estimated in preliminary experiments is about 25 mg/L) and forms a white precipitate (Sb_2O_3) when the solubility value is exceeded. In the presence of sulfide, Sb (III) could also precipitate as stibnite ($Sb2S_3$), a highly insoluble compound that has reddish color. The sludge was washed with medium to remove traces of soluble sulfide before the experiment and the bioassays were incubated for 30 days. After a while it was possible to distinguish a reddish precipitate, most likely stibnite. This precipitate was formed with at least two of the sludges used in the test (Rockteen and Eerbeek). The precipitate was visible in the Eerbeek bottles as reddish particles (Figure 4.16) and in the Rockteen ones the sludge changed its color from black to brown during this visual experiment. In addition, we had previously washed extensively with medium the sludges used in order to remove traces of soluble sulfide so these results were quite surprising.

The precipitation could be due to the release of sulfide from the microbial degradation of organic constituents in the sludge or remobilization of precipitated sulfide. The solubility product of Sb_2S_3 is extremely low (solubility product= 1.6×10^{-93} (Polack et al. 2009)) so the mobilization of precipitated sulfide is likely.

Figure 4.16. *Eerbeek bottle from the visual experiment showing; a) Initial appearance, b) After 7 days and c) Formation of black/reddish particles associated to sulfide reaction (stibnite and metastibnite) day 30.*

4.3.2. Screening of different inocula for their ability to reduce Sb (V) under anaerobic conditions

The first step in the bioreduction test was a screening trying all the available anaerobic inocula in order to find out if they were capable to reduce Sb (V) and how long it would take. Bottles lacking electron donor and other ones with hydrogen gas were prepared with the medium M2 (Table 3.4). Two speciation measurements were made at 5 and 30 days in order to check what was happening in the liquid phase. ICP-OES measurements were also made regularly to check if any antimony disappeared from the liquid phase. A different soluble antimony loss was measured (initially it was added 20 mg/L) with the speciation method depending on the sludge used. Results of the two speciation samples analyzed are shown in the next four figures. Results after 5 days in the Mahou bottles are not shown but there was no change in the total soluble antimony during those first 5 days and the Sb remained as Sb (V).

Figures 4.17 and 4.18 show the speciation of Sb determined for the different inocula after 5 days of incubation in endogenous assays (no e-donor) and in bioassays supplied with H_2. The total concentration of an antimony in all assays is lower than the concentration added (20 mg/L). This can be due to sulfide precipitation, biosorption, etc. After 5 days, the Sb added was still present as Sb (V) in all bioassays except for "Agua Nueva", which reduced Sb (V) to Sb (III) almost completely.

Figures 4.19 and 4.20 show the speciation of Sb determined for the different inocula after 30 days of incubation in endogenous assays (no e-donor) and in bioassays supplied with H_2. The soluble Sb in all the assays consisted almost exclusively of Sb (III), suggesting that these inocula were capable of reducing Sb (V). However, the total concentration of Sb in some assays ("Eerbeek", "Rockteen" and "INA" sludges) was considerably lower than the Sb concentration added initially (20 mg/L)._ Sb loss seems to be a bit higher in the H_2 bottles than in the endogenous ones. The loss of Sb could be due to the precipitation of Sb (III) as Sb_2S_3. The sludges were not washed to remove sulfide prior to set up these bioassays. . Sulfide has been previously shown to reduce Sb (V). In the presence of sulfide, the Sb (III) formed by chemical or biological reduction was precipitate forming highly insoluble Sb_2S_3 as discussed previously. This has been demonstrated in studies performed by *(Wang et al. 2013)* (Figure 1.1). The kinetics can not be compared because sulfide concentration was not measured during the tests performed in this research.

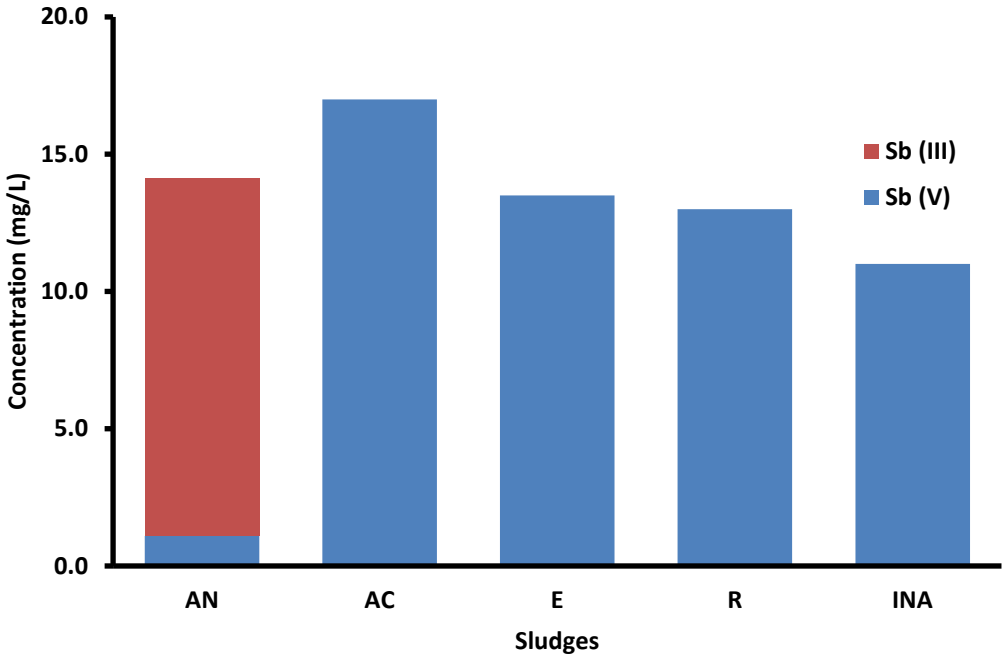

Figure 4.17. *Speciation samples after 5 days of incubation in bioassays lacking H_2 as electron donor (endogenous controls).*

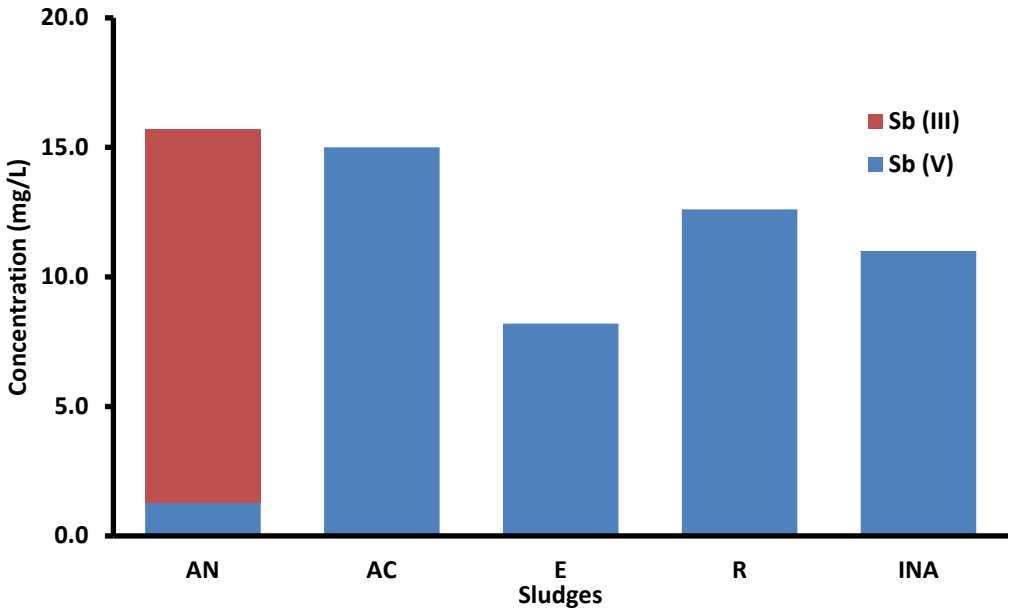

Figure 4.18. *Speciation samples after 5 days of incubation in bioassays with H_2 as electron donor.*

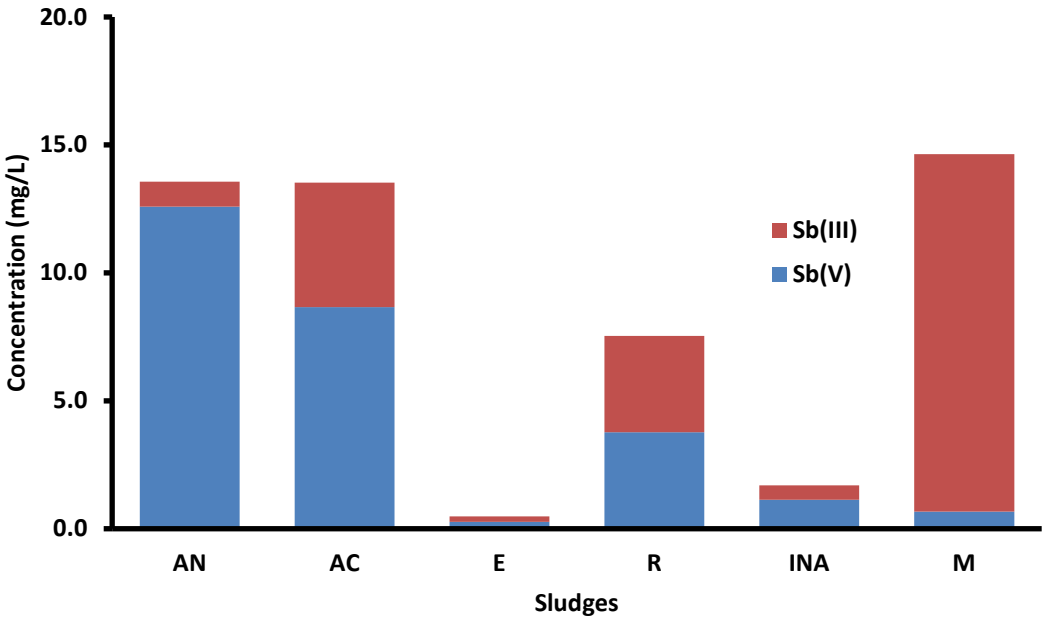

Figure 4.19. *Speciation samples after 30 days of incubation in bioassays lacking H_2 as electron donor (endogenous controls).*

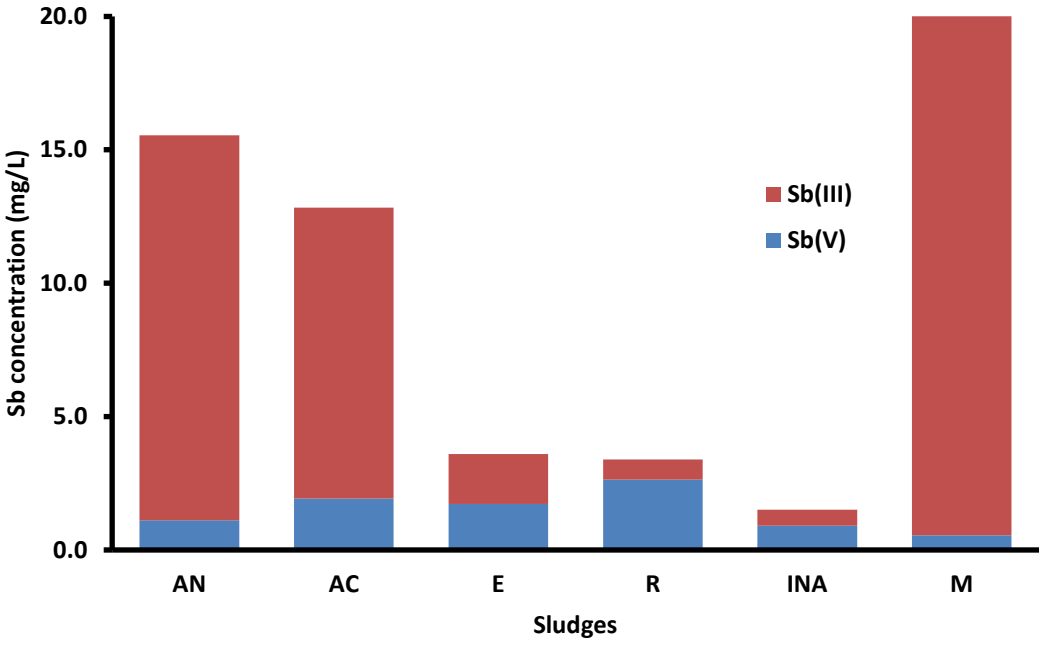

Figure 4.20. *Speciation samples after 30 days of incubation in bioassays with H_2 as electron donor.*

After comparing these results it was decided to work with the Agua Nueva sludge because of the fast reduction showed and with Mahou sludge because most of the Sb remained on the liquid phase and the aim was antimony reduction, not its precipitation.

4.3.3. Kinetics of Sb (V) reduction by Mahou and Agua Nueva inocula

The next experiments launched used Mahou sludge and Agua Nueva sludge and the aim was to evaluate the kinetics of Sb reduction by both sludges. Total antimony was measured just to ensure everything was working as expected and then the speciation was performed often to follow the reduction happening inside the bottles. The medium used in these tests was the M3 (Table 3.5). These sludges were not washed to remove traces of sulfide prior to inoculation. (Note: In further experiments, sludges were washed several times to remove sulfide traces).

Figure 4.21 shows that the total concentration of antimony remained constant in the Mahou tests during the 30 days of incubation as expected.

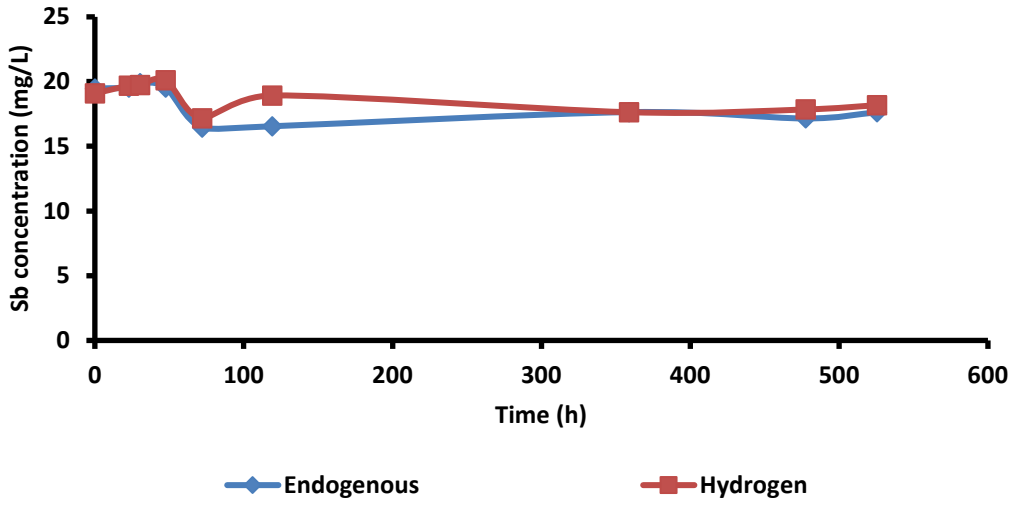

Figure 4.21. *Total antimony measured as a function of time during the incubation of Sb (V) with Mahou sludge in bioassays lacking electron donor (endogenous controls).*

Figures 4.22 and 4.23 show the speciation results obtained in the experiment with Mahou sludge in assays without H_2 (endogenous) and with H_2 gas (complete treatment) as a function of time. The graphs show a slow reduction of antimony and almost the same result when using or not hydrogen as electron donor. The Sb (V) conversion into Sb (III) happened in near stoichiometric proportions. The estimated rate of Sb (V) disappearance for the endogenous bottles in this "Mahou" test is $0.28\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$. For the ones with H_2 as electron donor the estimated rate of Sb (V) disappearance is $0.39\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$.

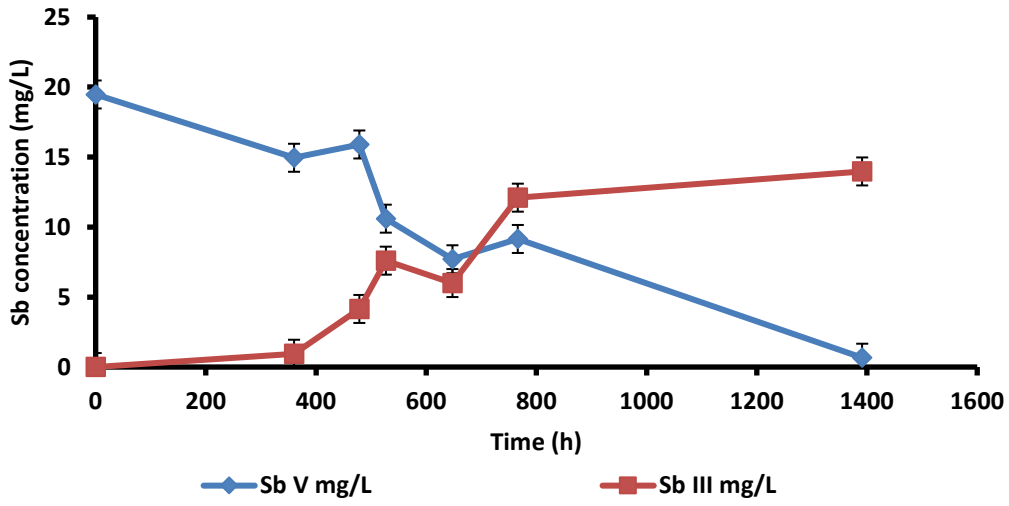

Figure 4.22. *Sb speciation as a function of time during the incubation of Sb (V) with Mahou sludge under anaerobic conditions in bioassays lacking electron donor (endogenous controls).*

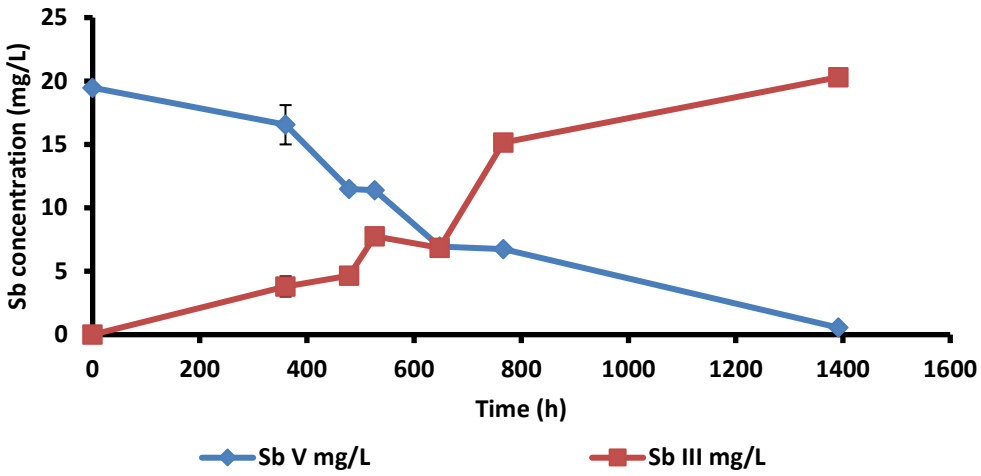

Figure 4.23. *Sb speciation as a function of time during the incubation of Sb (V) with Mahou sludge under anaerobic conditions in bioassays using H_2 as electron donor.*

A small loss of total soluble Sb was expected with Agua Nueva sludge but results showed that the total concentration of soluble Sb it remained constant during the experiment (Figure 4.24). As expected, Agua Nueva sludge worked faster than Mahou sludge. Also, the endogenous bottles worked slower than the bottles supplied with hydrogen as electron donor (Figures 4.25 and 4.26). The estimated rate of Sb (V) disappearance for the endogenous bottles is 3.28$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$ and for the bottles with H_2 is 10.2$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$.

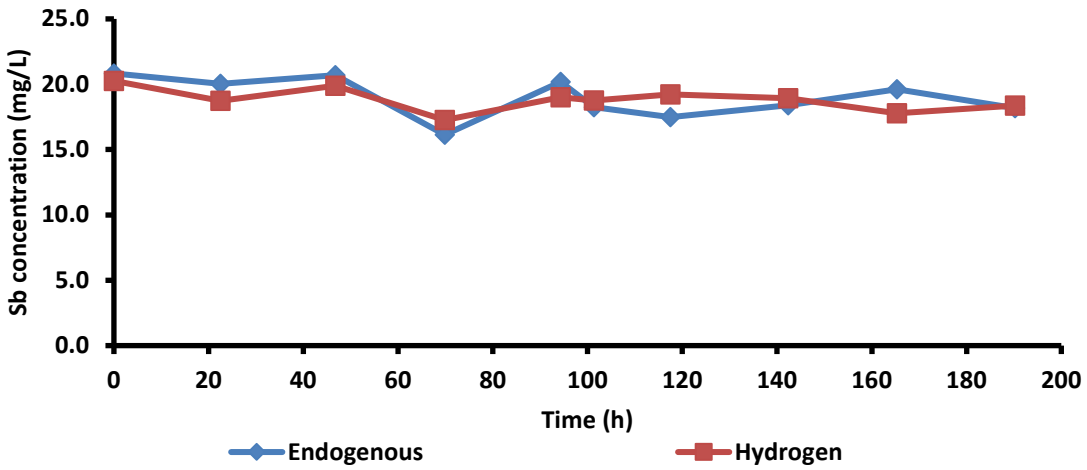

Figure 4.24. *Total soluble Sb concentration as a function of time during the incubation of Sb (V) with Agua Nueva sludge in anaerobic bioassays with/without H_2 as electron donor.*

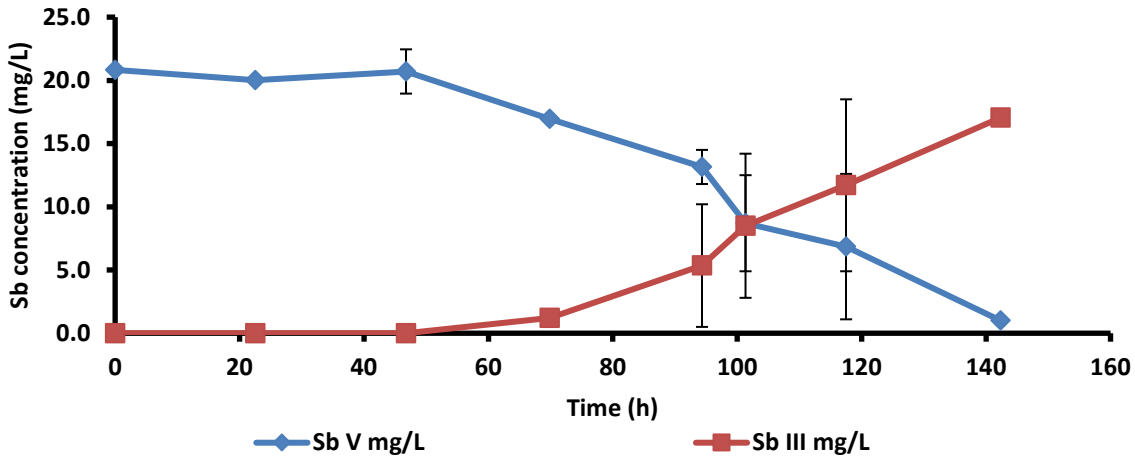

Figure 4.25. *Sb speciation as a function of time during the incubation of Sb (V) with Agua Nueva sludge in anaerobic bioassays lacking electron donor (endogenous control).*

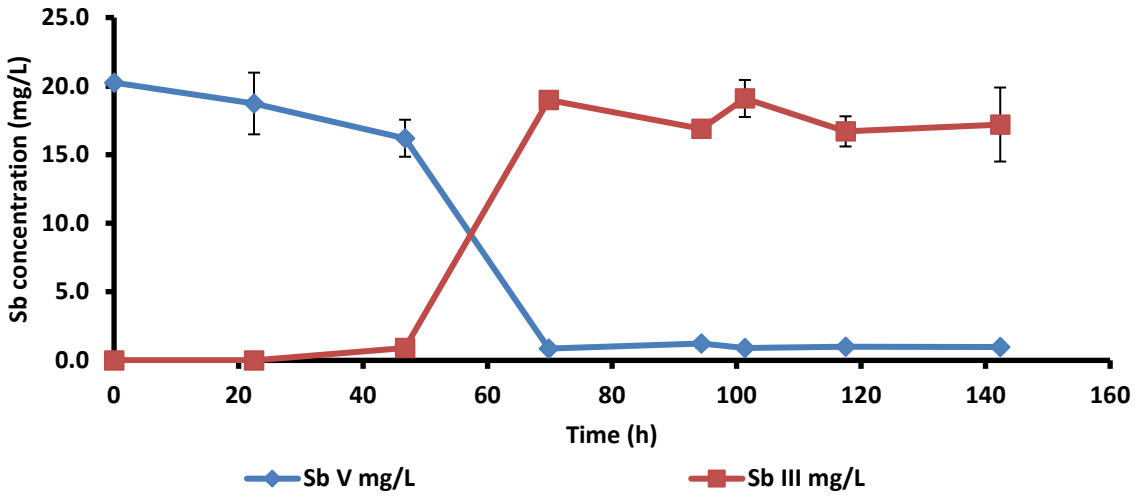

Figure 4.26. Sb speciation as a function of time during the incubation of Sb (V) with Agua Nueva sludge under anaerobic conditions in bioassays using H_2 as electron donor.

The high standard deviation values in Figure 4.25 are because one of the two endogenous bottles prepared worked a bit slower that the other. To be more precise, it seems like the results of this bottle were shifted one day from the results obtained from the other one.

In agreement with our results, Abin and Hollibaugh (2014) have recently reported that the bacterium MLFW-2, a member of the order Bacillales (phylum Firmicutes), was capable of using Sb (V) as a terminal electron acceptor for anaerobic respiration, resulting in the precipitation of Sb (III) as microcrystals of antimony trioxide. Abin and coworkers provided the first unequivocal evidence that a bacterium is capable of conserving energy for growth and reproduction from the reduction of Sb (V).

4.3.4. Kinetics of Sb (V) reduction by Agua Caliente, Rockteen and Eerbeek inocula

Another test was done using the remaining inocula (Agua Caliente, Rockteen and Eerbeek) to test their ability to reduce Sb (V) in more details than in the first screening. This test was only performed with H_2 as electron donor (endogenous treatments lacking H_2 were not included). There was a hypothesis that the reduction was chemical and not biological in the previous tests since we found that chemical reduction of Sb (V) could be done just by the sulfide. The inocula were washed with the medium used for the reduction tests (M3) and all the tests were prepared exactly the same as the Mahou and Agua Nueva ones from that point.

Figures 4.27, 4.28 and 4.29 show the concentration of total soluble Sb in the bioassays with the Agua Caliente, Rockteen and Eerbeek inocula, respectively, as a function of time. The losses of soluble Sb could be due to some remaining sulfur that makes Sb (III) precipitate as stibnite (Sb_2S_3).

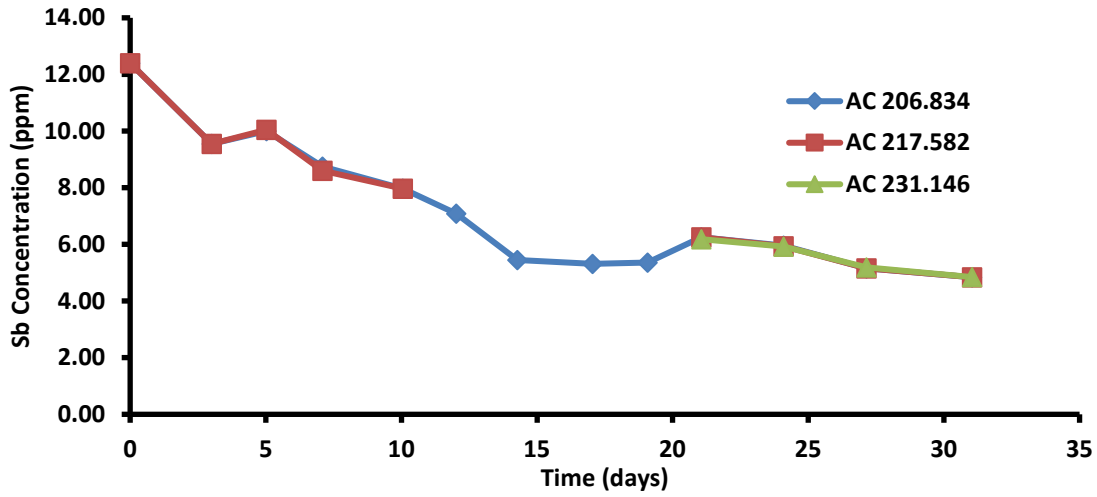

Figure 4.27. *Total soluble Sb concentration as a function of time during the incubation of Sb (V) with Agua Caliente sediments (washed) in anaerobic bioassays using H_2 as electron donor.*

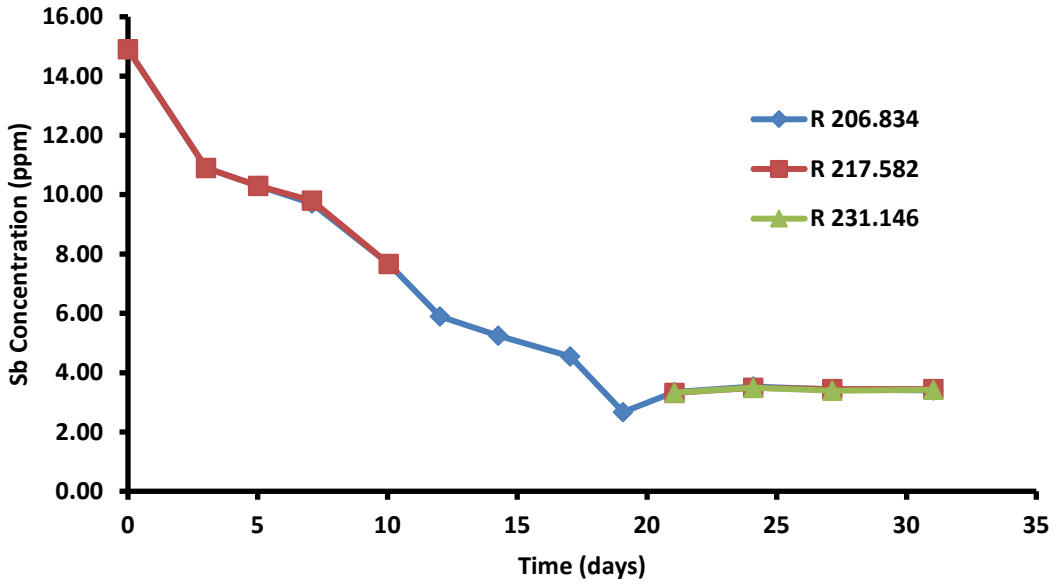

Figure 4.28. Total soluble Sb concentration as a function of time during the incubation of Sb (V) with Rockteen sludge (washed) in anaerobic bioassays using H_2 as electron donor.

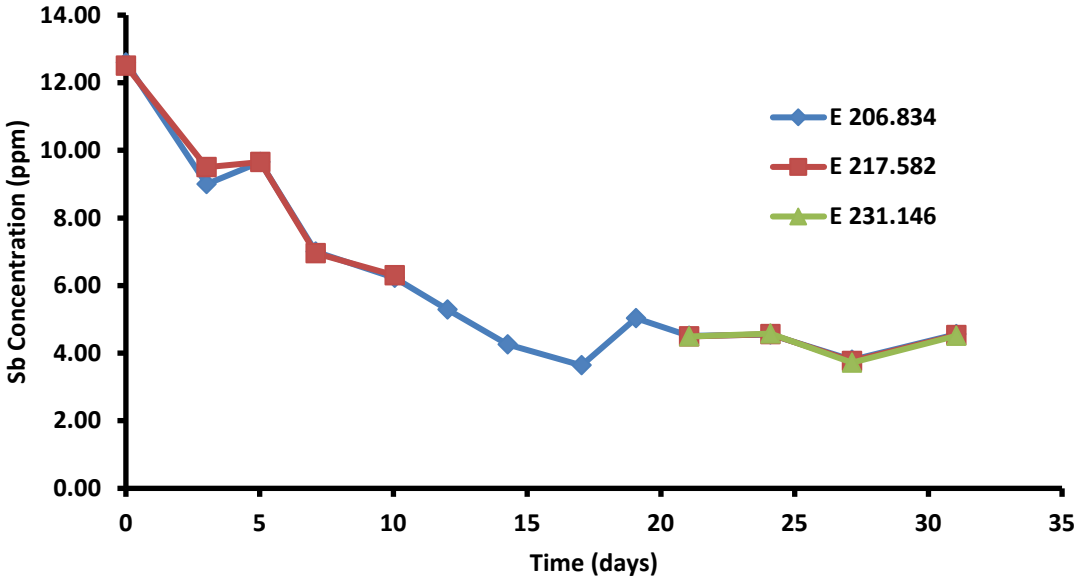

Figure 4.29. Total soluble Sb concentration as a function of time during the incubation of Sb (V) with Eerbeek sludge (washed) in anaerobic bioassays using H_2 as electron donor.

The speciation results obtained in the bioassays with “Agua Caliente”, “Rockteen” and “Eerbeek” inoculum show that the increase in the concentration of Sb (III) did not follow the magnitude of Sb (V) decrease (Figures 4.30-4.32). This fact gives strength to the hypothesis that sulfide released from the inoculum contributed to the precipitation of part of the Sb (III) as stibnite. The estimated Sb (V) disappearance rates are 0.56, 0.57 and 0.28$\frac{mg\ Sb\ (V)}{g\ VSS{\cdot}d}$ for “Agua Caliente”, “Rockteen” and “Eerbeek”, respectively.

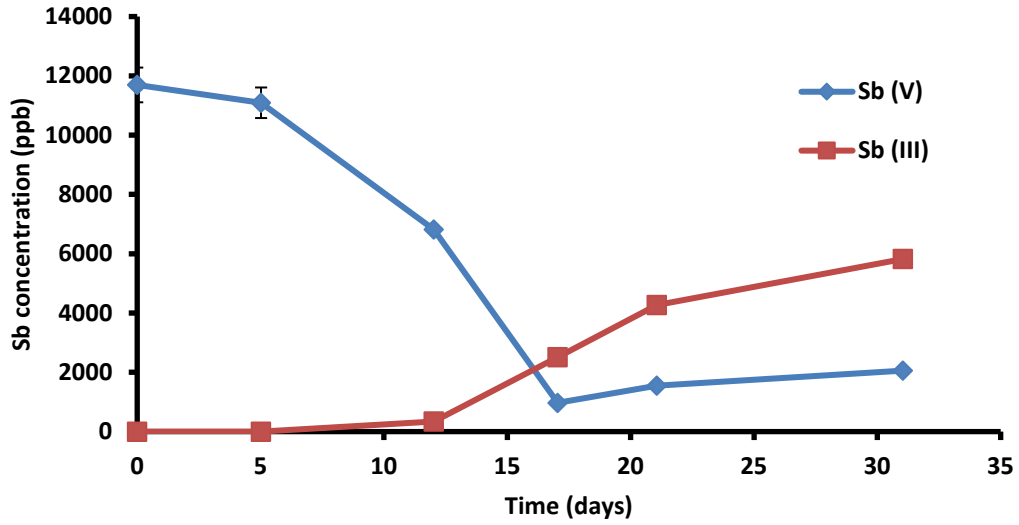

Figure 4.30. Sb speciation as a function of time during the incubation of Sb (V) with Agua Caliente sediments (washed) in anaerobic bioassays using H_2 as electron donor.

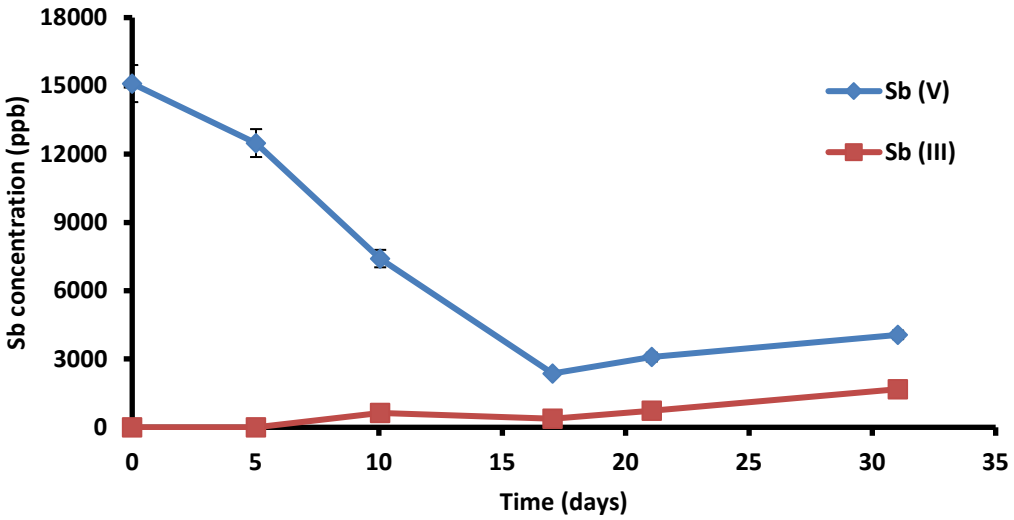

Figure 4.31. Sb speciation as a function of time during the incubation of Sb (V) with Rockteen sludge (washed) in anaerobic bioassays using H_2 as electron donor.

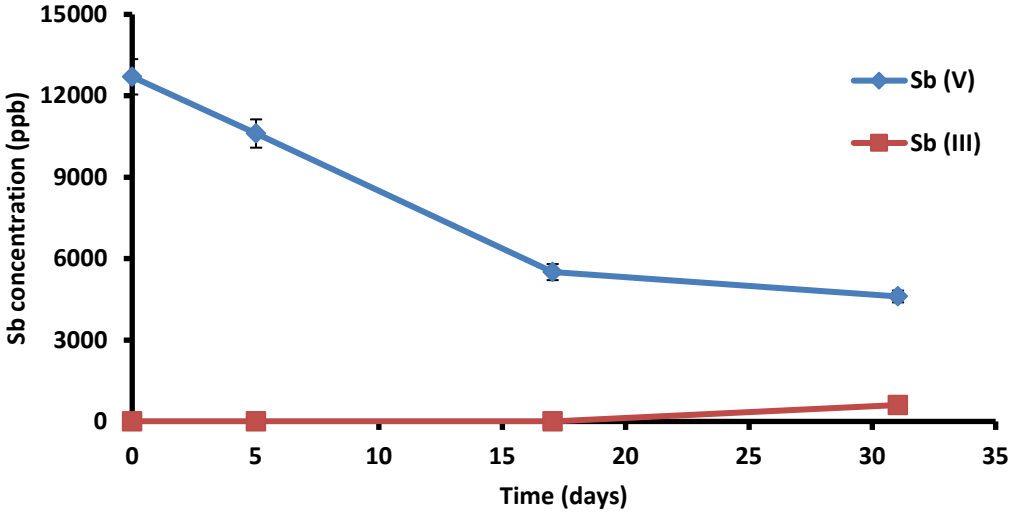

Figure 4.32. Sb speciation as a function of time during the incubation of Sb (V) with Eerbeek sludge (washed) in anaerobic bioassays using H_2 as electron donor.

5. CONCLUSIONS

- The HPLC-ICP-OES method developed in this study is a feasible alternative to the expensive HPLC-ICP-MS for the speciation of Sb when detection limits ranging 50-100 ug/L are acceptable.

- Sb (V) can be quantified reliably using ion chromatography (IC), however, interference by constituents in the bioassay media used precluded Sb (V) analysis in our studies. Sb (III) can not be quantified by IC because the species is not ionic over a broad pH range. The possibility of using a different column or different chromatographic conditions to analyze Sb (V) should be assessed. IC analysis is simple and fast.

- Sb (III) was found to be display higher microbial inhibition than Sb (V) towards the marine bacterium, *Aliivibrio vibrio* (Microtox), and anaerobic methanogenic microorganisms. The microbial toxicity of Sb (V) increased significantly with increasing exposure time, most likely due to the reduction of Sb (V) to the more toxic trivalent Sb.

- Anaerobic bioassays with different microbial inocula suggested that microorganisms can reduce Sb (V) to Sb (III). With the exception of one inoculum (Agua Nueva sludge), the loss of Sb (V) in bioassays with the inocula tested was slow (Mahou endogenous 0.28$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$, Mahou with H_2 0.39$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$, Agua Caliente with H_2 0.56$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$, Rockteen with H_2

0.57$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$, Eerbeek with H_2 0.28$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$, Agua Nueva endogenous 3.28$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$, Agua Nueva with H_2 10.2$\frac{mg\ Sb\ (V)}{g\ VSS \cdot d}$).

- The concentration of total soluble Sb decreased with time in most bioassays probably due to the reaction of Sb (III) with sulfide released from the inoculum and the ensuing precipitation of highly insoluble Sb_2S_3 (stibnite). This phenomenon was also observed in experiments using sulfide-free medium and sludges that were previously washed to remove traces of soluble sulfide. Some sulfide could be released during the incubation due to the endogenous degradation of the biomass or remobilization of precipitated sulfide.

- Future experiments should be designed to demonstrate unequivocally that microorganisms can reduce Sb (III) to Sb (V) in the absence of sulfide, and they should include suitable abiotic and killed-sludge controls. Further research should also consider the potential role of (biogenic) sulfide in the reduction of Sb (V) to Sb (III).

- Microbial reduction of Sb (V) in the presence of sulfide could provide the basis to develop a novel biotechnology to remove Sb from contaminated water.

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7. APPENDIXES

APPENDIX 1: Microtox acute toxicity test protocol.

Microtox is a fast toxicity assay that uses a strain of the symbiotic bacterium *Aliivibrio fischeri*, by measuring its bioluminescence at 490 nm. *A. fisheri* (the acute reagent) is sensitive to pH and temperature. Minimal pH effect occurs between 6 and 8. The Microtox system operates at below 10 ºC. Microtox Model: M500

Things needed

Sample (2x highest desired concentration), timer, recording sheet, and calibrated pipets (10 µL, 0.25 mL, 0.5 mL, 1 mL, 1.25 mL, 2.5 mL).

Analyzer set-up

1. Make sure Microtox is plugged in the outlet and reach proper temperature.
2. Place cuvettes in the reagent well and all rows (A though F).
3. Pipet 1 mL of Microtox Reconstitution solution into the reagent well cuvette.
4. Pipet 0.5 mL of Microtox Diluent into cuvettes in wells B1-B5, C1-C5, E1-E5, F1-F5.
5. Pipet 1.25 mL of Microtox Diluent into cuvettes in wells A1-A4, D1-D5. (Don't add diluent to A5)

Dilution set-up

1. Pipet 0.25 mL of Microtox Osmotic Adjustment solution (MOAS) into the cuvette in well A5.
2. Add 2.5 mL of sample into cuvette in A5. Mix using pipet by filling and dispensing 3-4 times.
3. Transfer 1.25 mL from A5 to A4. Mix using pipet.
4. Transfer 1.25 mL from A4 to A3. Mix using pipet.
5. Transfer 1.25 mL from A3 to A2. Mix using pipet.
6. Transfer 1.25 mL from A2 to D5. Mix using pipet.

7. Transfer 1.25 mL from D5 to D4. Mix using pipet.
8. Transfer 1.25 mL from D4 to D3. Mix using pipet.
9. Transfer 1.25 mL from D3 to D2. Mix using pipet.
10. Transfer 1.25 mL from D2 to D1. Mix using pipet.
11. Discard 1.25 mL from D1

Reagent preparation (They must be done fast and with precision)

1. Take a vial of Microtox reagent from the freezer. Remove and discard the seal and stopper.
2. Verify that pellet is placed on the bottom of the vial.
3. Take the cuvette containing the reconstitution solution from the reagent well. Pour quickly the reconstitution solution from the cuvette into the reagent vial.
4. Swirl the reagent vial 3-4 times on the benchtop. Pour the reagent vial into the cuvette in the reagent well.
5. Mix the reconstituted solution using a 0.5 mL pipet (use a new tip), by filling and dispensing 20 times. *A. fischeri* (the reagent) is active for 1 -2 hours after it has been reconstituted.
6. Pipet 10 uL of the reconstituted reagent into cuvettes in B1-B5, C1-C5, E1-45, F1-F5.
7. Mix the cuvettes with a 0.25 mL pipet, filling and dispensing 2-3 times
8. Wait 15 minutes for reagent to stabilize.

Recording data

1. After 15 minutes, place cuvette B1 in the read well and press SET button.
2. Read initial luminescence (t = 0 min) for cuvettes B1-B5, C1-C5, E1-E5, F1-F5 by placing each cuvette in the read well and pushing the READ button. Record values.
3. Start timer.

4. Using every time a new pipet tip, make 0.5 mL transfer, mixing 2-3 times with pipet after doing each transfer, from cuvette A1 to cuvettes B1 and C1; A2 to B2 and C2; A3 to B3 and C3, etc., Do the same for cuvette D1 into cuvettes E1, F1; D2 into cuvettes E2, F2, etc.
5. Read cuvettes B1-B5, C1-C5, E1-E5, F1-F5 at 5 min, 15 min, and 30 min. Record all data and process in Excel spreadsheet.

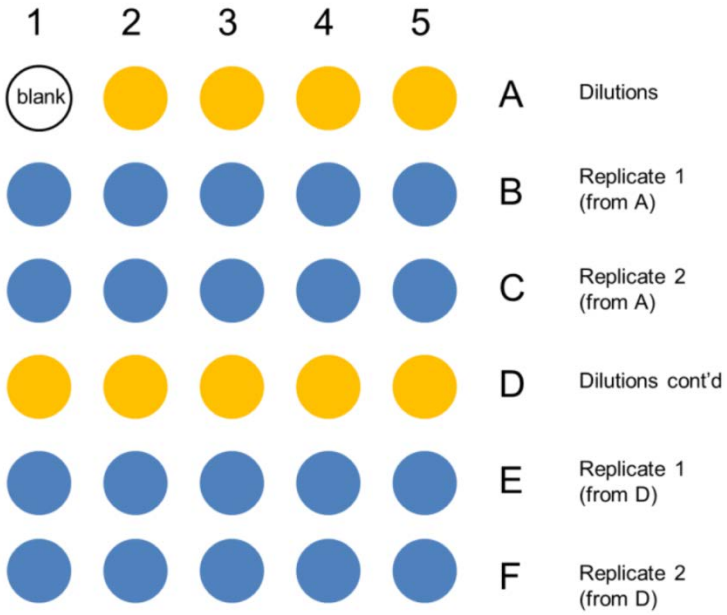
Notes:

Rows A and D are not "read".

A1 is a toxicant free control.

Read cuvettes:

Before adding toxicant (t = 0 min), and 5 min, 15 min, 30 min exposure.



Recording Sheet:

Time = _____ min

	1	2	3	4	5
B					
C					
E					
F					

Time = _____ min

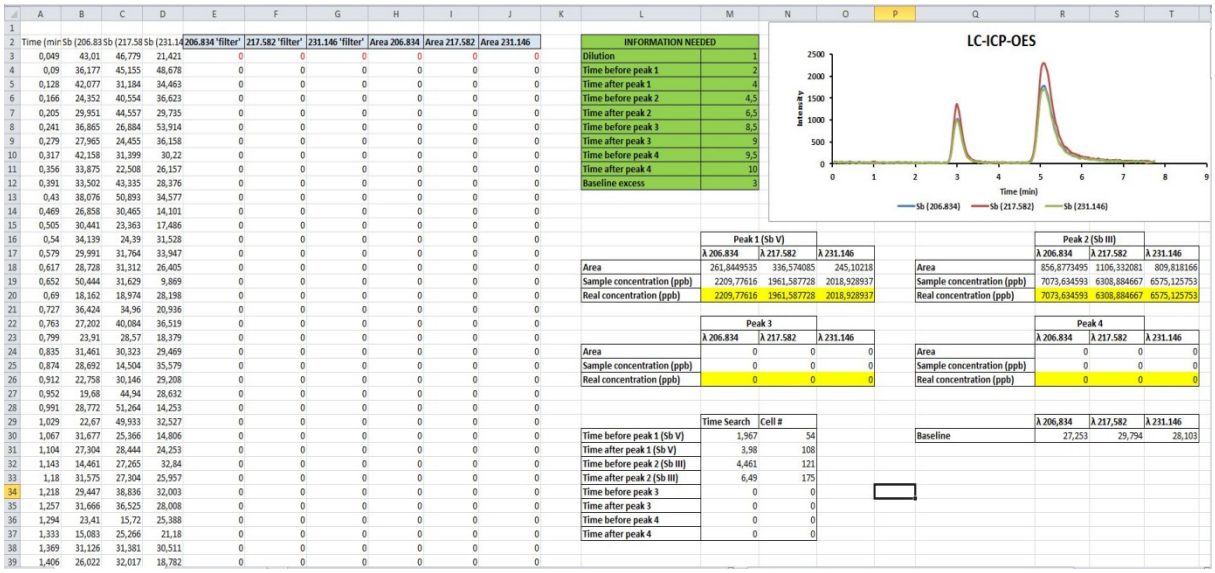
	1	2	3	4	5
B					
C					
E					
F					

Time = _____min

	1	2	3	4	5
B					
C					
E					
F					

APPENDIX 2: Integration Excel worksheet.

In order to integrate the chromatograms obtained from the LC-ICP-OES method an Excel worksheet was developed because if not, every sample would need a large amount of time to process. This Excel has six main parts which will be explained in this appendix in detail. To give a general idea of how this looks in the excel worksheet the next image is provided.



Time (min	Sb (206.83	Sb (217.58	Sb (231.14	206.834 'filter'	217.582 'filter'	231.146 'filter'	Area 206.834	Area 217.582	Area 231.146
0,049	43,01	46,779	21,421	0	0	0	0	0	0
0,09	36,177	45,155	48,678	0	0	0	0	0	0
0,128	42,077	31,184	34,463	0	0	0	0	0	0
0,166	24,352	40,554	36,623	0	0	0	0	0	0
0,205	29,951	44,557	29,735	0	0	0	0	0	0
0,241	36,865	26,884	53,914	0	0	0	0	0	0
0,279	27,965	24,455	36,158	0	0	0	0	0	0
0,317	42,158	31,399	30,22	0	0	0	0	0	0
0,356	33,875	22,508	26,157	0	0	0	0	0	0
0,391	33,502	43,335	28,376	0	0	0	0	0	0
0,43	38,076	50,893	34,577	0	0	0	0	0	0
0,469	26,858	30,465	14,101	0	0	0	0	0	0
0,505	30,441	23,363	17,486	0	0	0	0	0	0
0,54	34,139	24,39	31,528	0	0	0	0	0	0
0,579	29,991	31,764	33,947	0	0	0	0	0	0
0,617	28,728	31,312	26,405	0	0	0	0	0	0
0,652	50,444	31,629	9,869	0	0	0	0	0	0
0,69	18,162	18,974	28,198	0	0	0	0	0	0
0,727	36,424	34,96	20,936	0	0	0	0	0	0
0,763	27,202	40,084	36,519	0	0	0	0	0	0
0,799	23,91	28,57	18,379	0	0	0	0	0	0
0,835	31,461	30,323	29,469	0	0	0	0	0	0
0,874	28,692	14,504	35,579	0	0	0	0	0	0
0,912	22,758	30,146	29,208	0	0	0	0	0	0
0,952	19,68	44,94	28,632	0	0	0	0	0	0
0,991	28,772	51,264	14,253	0	0	0	0	0	0
1,029	22,67	49,933	32,527	0	0	0	0	0	0
1,067	31,677	25,366	14,806	0	0	0	0	0	0
1,104	27,304	28,444	24,253	0	0	0	0	0	0
1,143	14,461	27,265	32,84	0	0	0	0	0	0
1,18	31,575	27,304	25,957	0	0	0	0	0	0
1,218	29,447	38,836	32,003	0	0	0	0	0	0
1,257	31,666	36,525	28,008	0	0	0	0	0	0
1,294	23,41	15,72	25,388	0	0	0	0	0	0
1,333	15,083	25,266	21,18	0	0	0	0	0	0
1,369	31,126	31,381	30,511	0	0	0	0	0	0
1,406	26,022	32,017	18,782	0	0	0	0	0	0

INFORMATION NEEDED	
Dilution	1
Time before peak 1	2
Time after peak 1	4
Time before peak 2	4,5
Time after peak 2	6,5
Time before peak 3	8,5
Time after peak 3	9
Time before peak 4	9,5
Time after peak 4	10
Baseline excess	3

	Peak 1 (Sb V)		
	λ 206.834	λ 217.582	λ 231.146
Area	261,8449535	336,574085	245,10218
Sample concentration (ppb)	2209,77616	1961,587728	2018,928937
Real concentration (ppb)	2209,77616	1961,587728	2018,928937

	Peak 2 (Sb III)		
	λ 206.834	λ 217.582	λ 231.146
Area	856,8773495	1106,332081	809,818166
Sample concentration (ppb)	7073,634593	6308,884667	6575,125753
Real concentration (ppb)	7073,634593	6308,884667	6575,125753

	Peak 3		
	λ 206.834	λ 217.582	λ 231.146
Area	0	0	0
Sample concentration (ppb)	0	0	0
Real concentration (ppb)	0	0	0

	Peak 4		
	λ 206.834	λ 217.582	λ 231.146
Area	0	0	0
Sample concentration (ppb)	0	0	0
Real concentration (ppb)	0	0	0

	Time Search	Cell #
Time before peak 1 (Sb V)	1,967	54
Time after peak 1 (Sb V)	3,98	108
Time before peak 2 (Sb III)	4,461	121
Time after peak 2 (Sb III)	6,49	175
Time before peak 3	0	0
Time after peak 3	0	0
Time before peak 4	0	0
Time after peak 4	0	0

	λ 206,834	λ 217,582	λ 231.146
Baseline	27,253	29,794	28,103

Figure 7.1. Excel worksheet.

The first important part of the Excel worksheet is the green chart in the middle of the Figure 7.1. This numbers that have to be adjusted according to the compounds analyzed and the retention times for them have to be introduced by the user and basically they fix the dilution of the sample introduced, the times before and after every peak and the excess that wants to be applied to the medium base line value to select the peak points from the data obtained by the LC-ICP-OES analyzer. The next table shows the green chart and the common values used during the analysis performed in the reduction tests. How this input is used will be explained later in this appendix.

Table 7.1. *Input needed by the Excel worksheet.*

INFORMATION NEEDED	
Dilution	5
Time before peak 1	2
Time after peak 1	4
Time before peak 2	4,5
Time after peak 2	6,5
Time before peak 3	8,5
Time after peak 3	9
Time before peak 4	9,5
Time after peak 4	10
Baseline excess	3

The next part is the graph showing the chromatogram obtained from the speciation method. This is to ease the times input needed and to check easier if the times used are or not correct. The figure below shows the graph with a chromatogram obtained with the speciation method.

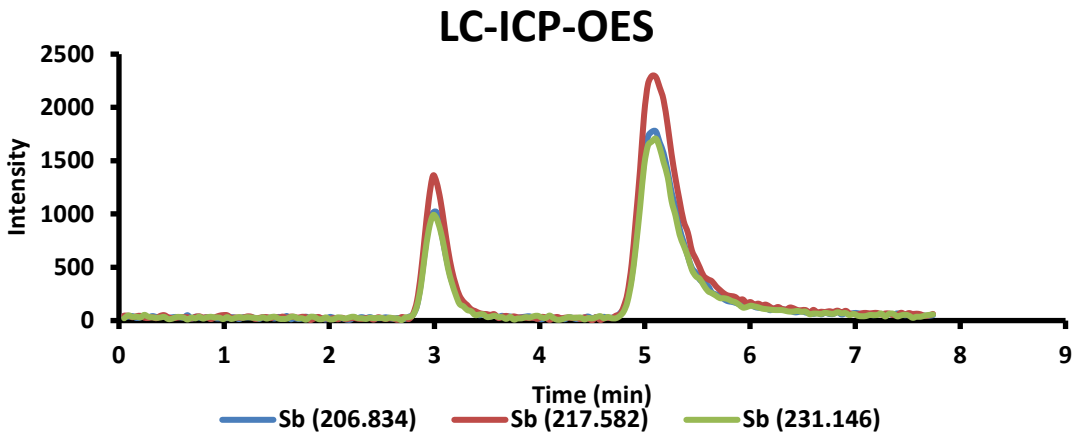


Figure 7.2. *Chromatogram obtained with the HPLC-ICP-OES speciation method.*

The next important part of the integration Excel is the chart where a filter is applied to the data obtained from the speciation method and the areas are calculated. The first four columns starting from the left (Figure 7.1) are the data obtained from the speciation method. This part has to be copied and pasted from to introduce the data that is going to be processed with this Excel worksheet. After that, the next three columns named as 'filter' are selecting from all the data the numbers that belong to the different peaks. The filters applied basically checks if the value of the intensity in the corresponding wavelength is greater than the baseline value multiplied by the baseline excess introduced in the green chart (Table 7.1) and, at the same time, the value belongs to one of the time lapses introduced in the green chart (Table 7.1) for any peak. If both conditions are true, then the filter returns the intensity minus the base lane value. If any of those conditions is false, there is another conditional checking the previous value in that column. If it is greater than zero, the intensity minus the base line is positive and the time is still inside a peak, it returns the value of that difference (Intensity minus baseline). This part allows us to obtain the areas of the las part of every peak because sometimes they fall lightly but we still want to integrate that part. Finally, if the conditions explained above are false, the value returned is zero.

When the data is filtered by the conditions explained above, it goes to the 'Area' columns where the area is calculated for each positive data in the 'filter' columns. The area is calculated with the trapeze formula since the data obtained with the speciation method is not continuous. The method measures every 2 seconds so this formula to calculate the area is going to calculate the exact area under the curve. The only condition programmed in the 'Area' columns is that the final value has to be greater than zero. This condition is programmed just to avoid a negative final value in the column because of the lack of time values since the formula is always calculating the time lapse between the points in the data by the difference of the time values. If there are no more time values, it would return a negative value and it could decrease the final area obtained. Instead of that, a zero value will return and the final area value will not change.

To ease the detection of the values integrated and the values that surpass the filter the cells with numbers greater that zero are automatically highlighted in yellow. With this, non-desired number surpasses the filter, the person using the Excel worksheet can notice it easily and fix it by changing either the baseline excess or the times to delimit the peak affected. Some rows showing the initial points (Figure 7.3) and some highlighted points (Figure 7.4) are showed below.

Time (min)	Sb (206.83	Sb (217.58	Sb (231.14	206.834 'filter'	217.582 'filter'	231.146 'filter'	Area 206.834	Area 217.582	Area 231.146
0,049	43,01	46,779	21,421	0	0	0	0	0	0
0,09	36,177	45,155	48,678	0	0	0	0	0	0
0,128	42,077	31,184	34,463	0	0	0	0	0	0
0,166	24,352	40,554	36,623	0	0	0	0	0	0
0,205	29,951	44,557	29,735	0	0	0	0	0	0
0,241	36,865	26,884	53,914	0	0	0	0	0	0
0,279	27,965	24,455	36,158	0	0	0	0	0	0
0,317	42,158	31,399	30,22	0	0	0	0	0	0
0,356	33,875	22,508	26,157	0	0	0	0	0	0
0,391	33,502	43,335	28,376	0	0	0	0	0	0
0,43	38,076	50,893	34,577	0	0	0	0	0	0
0,469	26,858	30,465	14,101	0	0	0	0	0	0
0,505	30,441	23,363	17,486	0	0	0	0	0	0

Figure 7.3. *Initial Data, 'filter' and 'Area' rows.*

2,686	19,776	32,445	4,156	0	0	0	0	0	0
2,722	15,174	26,628	28,489	0	0	0	0	0	0
2,758	36,779	15,171	33,399	0	0	0	0	0	0
2,796	54,001	62,131	52,625	0	0	0	0	0	0
2,834	141,58	186,731	141,149	114	157	113	2	3	2
2,87	346,974	436,438	320,338	320	407	292	8	10	7
2,906	628,352	775,468	597,691	601	746	570	17	21	16
2,945	899,022	1127,578	848,509	872	1098	820	29	36	27
2,985	1010,514	1358,299	987,806	983	1329	960	37	49	36
3,02	1015,903	1296,637	956,552	989	1267	928	35	45	33
3,057	873,133	1126,949	842,828	846	1097	815	34	44	32
3,092	687,707	901,849	681,951	660	872	654	26	34	26
3,128	514,352	668,077	491,552	487	638	463	21	27	20
3,165	366,566	474,305	353,921	339	445	326	15	20	15
3,202	272,381	336,478	242,149	245	307	214	11	14	10
3,237	188,509	235,378	157,815	161	206	130	7	9	6

Figure 7.4. *Some highlighted values obtained with the Excel worksheet.*

The third part that is going to be explained is the time search, cell number determination and medium baseline value calculation. This part searches for the numbers in the time column (Data introduced obtained from the LC-ICP-OES method) (Figure 7.3) and returns the most similar value to the one introduced in the green chart (Table 7.1) but always under that value. If the maximum value in the time column is much lower than the time searched, there is a filter to return zero instead of the maximum value of the time column to avoid later problems calculating the concentration. Then, there is another cell searching for the number of row where this value is contained. These columns are used later in the final area calculation. For the average baseline value all the data obtained from the LC-ICP-OES analysis excepting the data belonging to the different peaks is selected and an average value is calculated with all of them. These charts are shown in the tables below.

Table 7.2. Average baseline calculation.

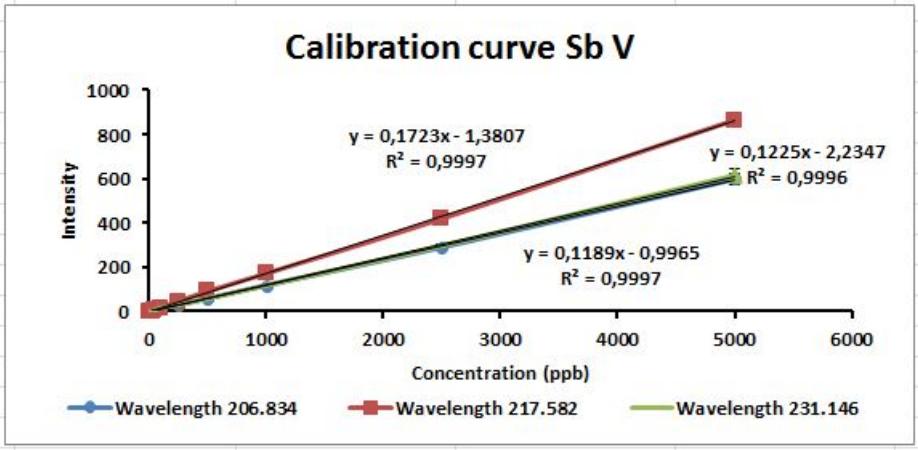
	λ 206,834	**λ 217,582**	**λ 231.146**
Baseline	27,253	29,794	28,103

Table 7.3. Time search and cell number chart.

	Time Search	**Cell #**
Time before peak 1 (Sb V)	1,967	54
Time after peak 1 (Sb V)	3,98	108
Time before peak 2 (Sb III)	4,461	121
Time after peak 2 (Sb III)	6,49	175
Time before peak 3	0	0
Time after peak 3	0	0
Time before peak 4	0	0
Time after peak 4	0	0

The next part is the calibration curves used and how to introduce them in the worksheet. The calibration curve is located in the second sheet of the document and consists in a ten point calibration that is automatically graphed and obtained (linear adjustment). There are 12 charts in this sheet in order to introduce a maximum of two complete calibration curves (one for each possible peak) and to calculate the standard deviations between the values introduced for each peak in the last four charts. The values calculated as a linear equation are returned to the first sheet of the document and then used to calculate the concentration equivalent to the area obtained in the last part that is going to be explained. In the next figure it is shown how the calibration charts and graph are put in the Excel worksheet.

Calibration curve Sb V (Peak 1)			
Concentration (ppb)	Wavelength 206.834	Wavelength 217.582	Wavelength 231.146
0	0	0	0
12,5	0	0	0
25	0	0	0
50	6,4162035	6,592672	5,6330135
100	14,836596	16,227114	15,449811
250	29,38702	44,77261375	28,156629
500	58,60288725	89,991086	56,54447325
1000	116,857919	171,0689905	116,905813
2500	288,9494938	418,9364353	296,007211
5000	597,5264565	864,556247	615,1345735



	Wavelength 206.834	Wavelength 217.582	Wavelength 230
Slope	0,118944853	0,172286372	0,122508936
Intercept	-0,996547754	-1,380747529	-2,234655871

Figure 7.5. One of the calibration curves used in the Excel worksheet.

The last part to be explained is the final results chart. In this chart the areas calculated in the first columns (Figure 7.3.) are selected depending on the time where they have been calculated and they are all summed in the corresponding peak. There is a little filter in this cell just to ensure that no negative areas are shown. If the number obtained by adding the corresponding areas is positive, this value is shown. In the concentration cell the area value is taken and, using the calibration curves explained before, this value is converted into a concentration one (showed as µg/L). There

is a conditional that also ensures that the concentration value is not negative (this could happen with low area values). This concentration is the sample's one. In order to calculate the real concentration the dilution introduced in the green table (Table 7.1) is used. The next table shows how these results are shown in the Excel worksheet.

Table 7.4. Final results table in the integration Excel.

	Peak 1 (Sb V)		
	λ 206.834	**λ 217.582**	**λ 231.146**
Area	261,8449535	336,574085	245,10218
Sample concentration (ppb)	2209,77616	1961,587728	2018,928937
Real concentration (ppb)	2209,77616	1961,587728	2018,928937

The final real concentration value is highlighted in yellow and corresponds to a single point in a reduction test. This method Excel took a lot of time to develop but also saved a lot of time while processing all the data obtained. At first every point could take around one hour to be analyzed but with this Excel now they only take a few seconds.

APPENDIX 3: List of products derived from this research

- Moreno-Andrade I., Regidor-Alfageme E., Ramos-Ruiz A., Field J.A., Sierra-Alvarez R. (2017). Microbial reduction of antimony by anaerobic microorganisms. 15th IWA World Conference on Anaerobic Digestion (AD-15) 17-20/10/2017. Beijing, China.

- Regidor E., Moreno-Andrade I., Field J.A., Sierra-Alvarez R. (2017). Anaerobic microorganisms are capable of reducing antimony (V) to Antimony (III). 10th World Congress of Chemical Engineering. 01-05/10/2017.Barcelona, España.

- Moreno-Andrade I., Regidor E., Ramos-Ruiz A., Field J.A., Sierra-Alvarez R. (2017). Toxicity of Antimony on anaerobic microorganisms. 90th Annual Conference & Exhibition, 03-05/05/2017. Phoenix, AZ, USA.

- Regidor E., Moreno-Andrade I., Field J.A., Sierra-Alvarez R. (2017). Anaerobic microorganisms are capable of reducing antimony (V). The University of Arizona Showcase. 22/02/2017, Tucson, AZ, USA.

- Moreno-Andrade I., Regidor E., Field J.A., Sierra-Alvarez R. (2017). Microbial inhibition by antimony compounds. 2017 AZ Water research Committee Building Our Sustainable water Future Workshop. 01/10/2017, Phoenix, AZ, USA.